OBSERVATIONS ON THE ADMINISTRATION OF DEHYDROASCORBIC ACID IN DIABETOGENIC DOSES TO THE ALBINO RAT.

Thesis

Submitted by

DEBABRATA RAYMAHASAYA, M.B.,B.Sc., (University of Calcutta).

for the degree of

DOCTOR OF PHILOSOPHY

University of Edinburgh.

EDINBURGH MAY, 1955.

ACKNOWLEDGEMENTS

I am deeply indebted to Dr. C.P. Stewart for suggesting the problem and for his advice and guidance in this investigation. I am grateful to Dr. S.K. Bhattacharya and Mrs. B. Iggo for all the help rendered. I am thankful to Dr. E. Gilchrist, Dr. J.A. Owen, Dr. J.S. Robson and Miss C.G. Thomson for their kind co-operation. I thank Miss B. Cousin, Mr. T. Allison, Miss M. Goodlad and others of this department for their help on numerous occasions.

Table of Contents

		Page
1.	Introduction	1
2.	Experimental, Materials and Methods	17
3.	Results	31
4.	Discussion	46
5.	Summary and Conclusions	54
6.	Appendix	56
7.	Bibliography	58

- - - - -

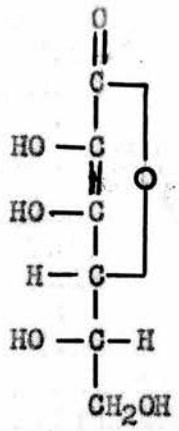
INTRODUCTION.

Szent-Györgyi (1) during his investigations of biological oxidation-reduction systems isolated a crystalline substance, highly reducing in its properties, from the ox adrenal gland, cabbage and orange. This substance was acid in nature, optically active and gave the colour reactions characteristic of the sugars. He called this substance hexuronic acid and suggested the chemical formula $C_6H_8O_6$. Tillmans (2) from his investigations on vitamin C suggested the possible identity of vitamin C with hexuronic acid. King and Waugh (3) and Svirbely and Szent-Györgyi (4) showed that the crystalline substance hexuronic acid was antiscorbutic in the guinea pig. Szent-Györgyi and Haworth (5) renamed hexuronic acid Ascorbic Acid. Harris and Ray (6), Hirst and Zilva (7) confirmed the antiscorbutic property of ascorbic acid.

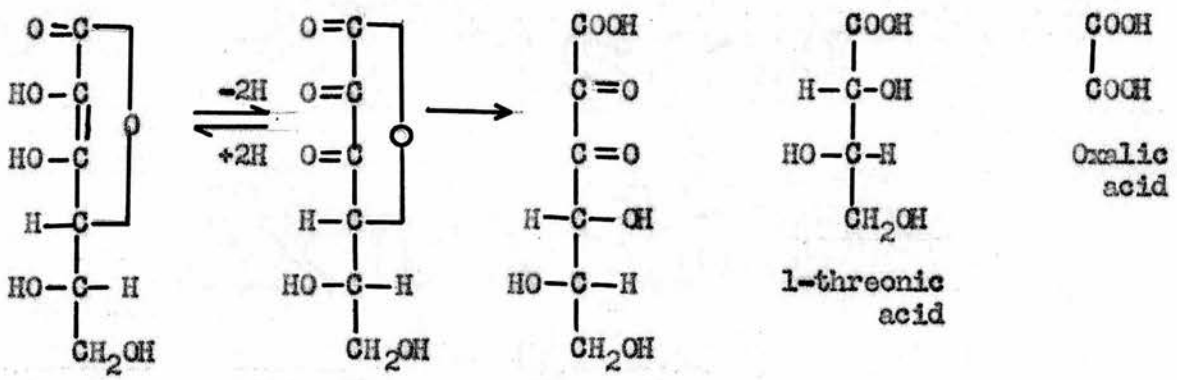
Reichstein, Grüssner and Oppenauer (8) and Ault, Baird, Carrington, Haworth, Herbert, Hirst, Percival, Smith and Stacey (9) synthesized ascorbic acid in the laboratory from xylosone. Over the years our knowledge of vitamin C has grown and it is difficult to assess the importance of the contributions of the many workers in the field.

Herbert, Hirst, Percival, Reynolds and Smith (10) worked out the structure and chemical properties of ascorbic acid on samples isolated from natural sources by Szent-Györgyi before the synthesis of ascorbic acid was made. They showed that ascorbic acid (ASA) had the structure as shown below/

below :-



In this detailed study of the chemistry, Herbert et al. showed that ascorbic acid behaves as a weak organic acid and is very readily oxidized. The stages of oxidation in the breakdown of the ascorbic acid molecule are as follows :-



Herbert et al. showed that the oxidation of ascorbic acid occurs in two well defined stages. The first stage in the oxidation involved in effect the addition of two hydroxyl groups to a double bond giving rise to a hydrated product,/

product, neutral in character and behaving as a lactone of a monobasic hydroxy acid. No disintegration of the molecule takes place during this process as the product can be converted, readily and quantitatively, into ascorbic acid by reducing agents such as hydrogen sulphide (7,11) or hydroiodic acid. This reversible dehydrogenated product dehydroascorbic acid (DHA), when newly formed, loses the property of selective absorption characteristic of ascorbic acid, and when kept in aqueous solution at room temperature it mutarotates and the property of selective absorption reappears. The product of the mutarotation is 2:3-diketo-l-gulonic acid (DKA) and dehydroascorbic acid is a lactone of 2:3-diketo-l-gulonic acid with two of its carboxyl groups hydrated. Ascorbic acid itself is the reduced form of this hydrated lactone. Herbert et al. suggested that this transformation into 2:3-diketo-l-gulonic acid involves the opening up of the lactone ring of the ascorbic acid molecule and conversion into an open chain six carbon compound. From 2:3-diketo-l-gulonic acid, reformation of ascorbic acid is possible, in vitro, under drastic treatment by lactonisation followed by reduction. When 2:3-diketo-l-gulonic acid is oxidized the six carbon chain is broken down with the formation of oxalic and l-threonic acids.

L-Xyloascorbic acid, the only naturally occuring form of ascorbic acid, so far known, is widely distributed in both the animal and plant kingdom. Man, primates and guinea pigs need vitamin C supplements in their diets, to remain free from the deficiency disease scurvy, which the lack of ascorbic acid produces. Synthesis of ascorbic acid in the animal body does occur. In the case of rats, for which ascorbic acid is not essential as a vitamin, when stimulated/

stimulated to synthesize, by the administration of chloretone, they most probably do so at the expense of glucose. King and his collaborators (12, 13,14), in tracer studies with glucose-C^{14}, have shown that D-glucose is almost certainly a precursor of ascorbic acid in the animal body. This synthesis most probably takes place through the intermediate formation of glucuronic acid. Isherwood, Chen and Mapson (15) in their extensive studies on the synthesis of ascorbic acid in plants and animals have put forward evidence to show that the sequence of events in the synthesis is probably as follows :

(1) D-glucose ⟶ D-glucurono- γ-lactone

⟶ L-gulono- γ-lactone ⟶ L-ascorbic acid

(2) D-galactose ⟶ D-galacturonic acid methyl ester

⟶ L-galactono- γ-lactone ⟶ L-ascorbic acid

Reid (16) summarises the functions of ascorbic acid, as known to-day, as follows :-

1. "It regulates the colloidal condition of intercellular substances having collagen or related substances as basic constituents. This is its most definitely established function.
2. It protects hydrogen carriers, thereby preventing injurious oxidations by acting as an antioxidant. An example of this type of action is to be found in its relations with α-tocopherol.
3. It promotes certain types of oxidation such as that of fatty acids and the production of the oxy type of adrenal cortical hormones (70).
4. It preserves a proper balance between different enzyme systems; the oxidation/

oxidation-reduction potentials are thus maintained at certain levels.

5. It plays a role in the formation of desoxyribo-nucleic acid, a substance held to control the physical-chemical properties of the nuclei.

It is always tempting to the scientific mind to imagine an active agent - vitamin, hormone or the like - as possessing a single fundamental activity, no matter in how many superficial ways this may ultimately appear. A list of activities such as that given above is, consequently, an indication that the real mode of action of ascorbic acid is still unknown and that it remains necessary to search for the common factor in these diverse manifestations. Not only is our ignorance of the physiological action of ascorbic acid profound, our fundamental knowledge of its mode of disposal or destruction in the animal body is also limited. Szent-Györgyi (17) showed the presence of an enzyme, "ascorbic acid oxidase" capable of catalyzing the direct oxidation of ascorbic acid with molecular oxygen in the plant tissues. The counterpart of this enzyme in the animal body has so far eluded search. Enzymes responsible for indirect catalysis of the oxidation of ascorbic acid to dehydroascorbic acid have been demonstrated. Keilin and Hartree (18) have shown that ascorbic acid is rapidly oxidised by cytochrome oxidase along with cytochrome c. Enzymes catalyzing the conversion of dehydroascorbic acid into diketogulonic acid have not been demonstrated so far, though the conversion is generally accepted (19,20). Enzymes catalyzing the cleavage of the diketogulonic acid molecule are, so far, not known.

Penney and Zilva (20) from their studies found that dehydroascorbic acid tends to be converted to diketogulonic acid with comparative ease, both in the presence/

presence and absence of oxygen, the rate being dependent on the pH of the medium. At neutrality or at the normal pH of the tissues the conversion was very rapid. Diketogulonic acid once formed cannot be reconverted *in vivo* (19,21) and it was antiscorbutically inert. They considered the possibility of the following pathway of metabolism

$$ASA \rightleftharpoons DHA \longrightarrow DKA$$

mentioning that under physiological conditions the concentration of dehydroascorbic acid at any one time was very small and, in consequence, the loss of ascorbic acid through the conversion of dehydroascorbic acid into diketogulonic acid would be very small.

In the same year, Rosenfeld (22) questioned the transformation of dehydroascorbic acid into the irreversible non-oxidative product diketogulonic acid in the degradation of ascorbic acid in the body. According to him the dehydroascorbic acid was but a fraction of the total ascorbic acid content of the living tissues, owing to the natural system for the protection of this vitamin and the presence of excessive amounts of glutathione. The mechanism of degradation consisted first, in the formation of an intramolecular stabilization product from dehydroascorbic acid which, in the presence of phosphate and at pH 7, was broken down quantitatively into oxalic acid through a precursor possibly oxalyl threose.

Damron, Monier and Roe (23) have suggested that the normal catabolism of ascorbic acid does not take place solely through the conversion to dehydroascorbic acid and diketogulonic acid. On administration of large doses, the catabolism may proceed along the pathway suggested by Penney and Zilva/

Zilva (20). Burns, Burch and King (24), from a tracer study of the metabolism of ascorbic acid in the guinea pig with l-C^{14}-l-ascorbic acid, have come to the conclusion that the conversion of ascorbic acid to CO_2 is a main pathway in its metabolism. The excretion of large amounts of oxalates in the urine of the animals in these experiments, indicated that oxalic acid is an end product of the metabolism. Burns, Mosbach and Schulenburg (25), from their studies in the rat with labelled ascorbic acid, arrived at similar conclusions with regard to the metabolic fate of ascorbic acid.

Monier and Weiss (26,27,28) have demonstrated an increased excretion of dehydroascorbic acid and diketogulonic acid in albino rats under conditions of stress.

Lamden and Chrystowski (29) observed increased excretion of urinary oxalates in man on ingestion of four or more grams of ascorbic acid.

Clayton, McSwinney and Prunty (30), from their metabolic studies with pure preparations of dehydroascorbic acid in man and guinea pigs, found that dehydroascorbic acid on absorption into the system was partly reduced to ascorbic acid and partly converted to oxidation products. The amount oxidised could be recovered, as "Roe and Kuether" material, only in part, a greater portion being lost as other oxidation products, they argue, possibly oxalate and carbon dioxide.

The outstanding property of ascorbic acid is the relative ease with which it may be reversibly oxidised. It can be reversibly oxidised by a number of substances such as halogens, charcoal (31), and benzoquinone. The relation between the chemical and biological processes leading to the oxidation/

oxidation of ascorbic acid has engaged the attention of many investigators. Both copper and iron salts have been shown to catalyze the oxidation (32, 33) and the oxidative activity of many tissues may be ascribed to this cause. The working out of the possible mechanism of copper catalyzed oxidation by Weissberger and LuValle (34) with the postulation of a semiquinone form, lends support to the view of the occurence of monodehydroascorbic acid in the tissues. The question of the existence of this substance in the tissues and its importance in metabolism remain to be settled. Other substances such as haemochromogens (35) were shown to be good catalysts. Stotz, Harrer, Schultze and King (36) observed a slow oxidation of added ascorbic acid by guinea pig liver brei and attributed this to the presence of the cytochrome system. Giri (37) observed, with rat liver homogenates, that the factors causing oxidation of ascorbic acid were present mostly in the nuclear fraction whereas all the other fractions inhibited the oxidation. As a link in the chain of this oxidation-reduction reaction the presence of dehydroascorbic acid in the tissues appears to be a possibility. Kinsey (38) confirmed the presence of about 3 mg/100 ml. of dehydroascorbic acid in the normal aqueous humour of the rabbit. Stewart, Horn and Robson (39) obtained evidence for the presence of significant amounts of dehydroascorbic acid in human plasma.

Szent-Györgyi (1) showed that in minced rat kidney reversibly oxidised hexuronic acid could be reduced by fixed -SH and glutathione. Hopkins and Morgan (40) followed this up with quantitative experiments and observed that when glutathione and ascorbic acid were together in the presence of ascorbic/

ascorbic acid oxidase, glutathione itself underwent oxidation thus sparing ascorbic acid. They observed the reduction of dehydroascorbic acid by glutathione as well, but noted that it was a slow process. Borsook, Davenport, Jeffreys and Warner (19) showed that dehydroascorbic acid added to minced or intact tissues from rats was rapidly reduced and concluded that the reducing mechanism possibly involved glutathione and fixed -SH groups. They did not find any evidence for enzymic reduction. The reduction of dehydroascorbic acid by slices or sections of surviving scorbutic guinea pig tissue was demonstrated by them. Schultze, Stotz and King (41) observed, with guinea pig tissues, that dehydroascorbic acid was reduced by a number of tissues, liver, muscle, small intestine, blood and erythrocytes. They noted an increased capacity for reduction after heat coagulation in the liver, muscle, whole blood and red cells. Blood plasma, heated or unheated, failed to reduce dehydroascorbic acid. Addition of iodoacetate, arsenite or alloxan inhibited the reduction completely. They concluded that glutathione and fixed -SH compounds of tissues are responsible for the reduction of added dehydroascorbic acid and denied the existence of any enzymic reduction. They suggested that the reduction of dehydroascorbic acid by animal tissues depends greatly upon the participants in the reversible reaction

$$\text{dehydroascorbic acid} + 2\ \text{-SH} \rightleftharpoons \text{ascorbic acid} + \text{-S-S-}.$$

This, as an enzymic reaction, has not yet been demonstrated in animal tissue. Recently, glutathione reductase, an enzyme catalyzing the reduction of oxidised glutathione in the presence of dehydrocoenzyme II has been shown to exist/

exist in plant tissues (42,43) and in the rat liver (44). The enzyme catalyzes the reaction

$$GSSG + TPNH_2 \longrightarrow 2GSH + TPN,$$

the reverse of which could not be demonstrated. A possible scheme of hydrogen transfer involving ascorbic acid, glutathione and coenzyme II in animal tissues

$$GSSG + TPNH_2 \longrightarrow 2GSH + TPN$$

Dehydroascorbic acid + $2GSH \rightleftharpoons$ ascorbic acid + GSSG has been considered. That such a system possibly exists is realised when we consider that neither dehydroascorbic acid nor oxidised glutathione remain in the tissues.

Johnson and Zilva (45) observed that when dehydroascorbic acid was ingested in man, it appeared as reduced ascorbic acid in urine. Fox and Levy (46) from their studies on the guinea pig confirmed the antiscorbutic property of dehydroascorbic acid. They found that dehydroascorbic acid was reduced to ascorbic acid which was stored to a small extent in the livers of the experimental animals. Roe and Barnum (47) made a quantitative study of the physiological activity of dehydroascorbic acid when administered orally or parenterally to guinea pigs. They could not detect any storage of vitamin C either in the oxidised or in the reduced form in the tissues. They found that dehydroascorbic acid was reduced in the blood of guinea pigs, rats and man and that this reduction was due to an enzyme. Dehydroascorbic acid was much less potent in its antiscorbutic property than the reduced form of the vitamin and this antiscorbutic property was due to the enzymic/

enzymic reduction in blood. Borsook et al. (19) confirmed the antiscorbutic property of dehydroascorbic acid. They found that, on ingestion of dehydroascorbic acid, there was an increased concentration of ascorbic acid in blood plasma and ascorbic acid was excreted in large quantities in the urine. Dehydroascorbic acid was rapidly reduced in the tissues of the animal body.

Gould and Shwachman (48) in their investigations on methods of bioassay of antiscorbutic substances with guinea pigs concluded that the antiscorbutic potency of dehydroascorbic acid was about 80 per cent activity of the pure vitamin. Penney and Zilva (20), from their metabolic studies on the guinea pig, observed that on administration of dehydroascorbic acid by intramuscular injection it was partly reduced by the organism to ascorbic acid and partly converted to diketogulonic acid. The ascorbic acid entered the tissues. Diketogulonic acid was mainly excreted in the urine. They suggested that when large quantities of dehydroascorbic acid are introduced into the circulation it is mainly reduced in the liver. When ingested, dehydroascorbic acid remained stable and stationary for sometime in the stomach before being gradually absorbed. It was reduced to ascorbic acid in the tissues of the stomach while being absorbed as shown by the increased concentration of ascorbic acid. The dehydroascorbic acid that escaped into the small intestine was converted to diketogulonic acid owing to the alkaline condition of the medium. Todhunter, McMillan and Ehmke (49), in their investigations on the extent of utilisation of dehydroascorbic acid in the human system, came to the conclusion that when dehydroascorbic acid was ingested, the levels of reduced ascorbic acid in the blood serum and urine were/

were comparable to those obtained when the reduced form of the vitamin was taken, indicating satisfactory utilisation. They found no consistent evidence for the presence of dehydroascorbic acid in blood or urine following the ingestion.

Langham (50) has shown that, in the rabbit, intravenous administration of dehydroascorbic acid leads to a greater concentration of the reduced form in the aqueous humour, than that obtained on administration of ascorbic acid by the same route. He suggests that the increased accumulation of ascorbic acid in the aqueous humour involved the reduction of dehydroascorbic acid by the lens and other intraocular structures. Whereas in the cat, he found, that on intravenous administration, dehydroascorbic acid or ascorbic acid appeared to pass into the eye at the same and a much slower rate. Kinsey (38) observed that dehydroascorbic acid was reduced by solutions of glutathione or crushed lenses but not by intact lenses in solution or in eyes which had been removed. He found that the injection of dehydroascorbic acid into the posterior chamber did not lead to any increase of ascorbic acid content in the anterior chamber. He confirmed the findings of Langham in the rabbit following intravenous administration of dehydroascorbic acid but explained it on the basis of the rise in concentration of ascorbic acid in blood plasma.

Patterson and Mastin (51) reported that ten minutes after an intravenous injection of dehydroascorbic acid, the ascorbic acid level in the rat brain was markedly raised from a rapid intracellular reduction of dehydroascorbic acid in the brain. A dose of 200 mg/Kg. DHA given intravenously/

venously in the rat more than doubled the ascorbic acid content of the brain, whereas the administration of a similar dose of ascorbic acid did not affect the ascorbic acid level. With repeated doses of dehydroascorbic acid the level of ascorbic acid in the brain tends to rise but the increase becomes progressively smaller due to the accumulation of ascorbic acid from reduction.

In man, Matusis (52) found the capacity to reduce dehydroascorbic acid to ascorbic acid was greatest in the liver. The other organs in order of their capacity to reduce are the kidney, adrenal, pancreas and intestine. This capacity is diminished in certain of the organs of people dying of prolonged illnesses, while the pancreas and adrenals retained it to the full. Similar reduced capacities were observed in the organs of scorbutic or tuberculous guinea pigs, white rats in experimental intestinal obstruction and white mice after chronic poisoning with neoarsphenamine.

Damron, Monier and Roe (23), in their metabolic study could recover as ascorbic acid 38 ± 16 per cent after one hour and 33 ± 20 per cent after two hours, the injected dose being approximately 25 mg/Kg. body weight of dehydroascorbic acid given in young guinea pigs. Of this dose, 19 ± 15 per cent and 8.3 ± 14 per cent were recovered as DHA while 18 ± 9 per cent and 9.5 ± 1.6 per cent were converted to DKA after the same intervals.

Following the isolation of dehydroascorbic acid and its derivatives by Kenyon and Munro (53) and by Pecherer (54) in the solid state, de Ritter, Cohen and Rubin (55), from their investigations on the physiological availability on ingestion in man, concluded that dehydroascorbic acid and dehydroascorbic/

ascorbic acid methanolate were well utilised. They did not find any dehydroascorbic acid in the urine following ingestion.

Panteleeva (56) observed that *in vitro* under physiological conditions of pH and temperature dehydroascorbic acid rapidly diffuses into human erythrocytes, the speed of this diffusion is much greater than that of ascorbic acid. Dehydroascorbic acid diffuses also into the erythrocytes in blood from the horse, cat, rabbit, dog and goose, but at a slower rate. It is partially reduced inside the red corpuscles to ascorbic acid and he suggested that the movement of ascorbic acid between erythrocytes and the plasma occurs in the form of dehydroascorbic acid. Lloyd (57) reported intracellular reduction in erythrocytes of dehydroascorbic acid added to defibrinated human blood. Lloyd and Parry (58) have estimated the capacity of human erythrocytes for reducing dehydroascorbic acid and suggest that the rate of reduction of DHA to ASA is similar to the rate of transformation of DHA to DKA. From their attempts to establish a ceiling level of reduced ASA in erythrocytes they suggest that the power of erythrocytes to reduce DHA is "limited by kinetic rather than by stoichiometric factors".

Bhattacharya (59) has shown that dehydroascorbic acid undergoes reduction in the erythrocytes of the rabbit, both *in vitro* and *in vivo*. A considerable amount of the dehydroascorbic acid appears to be converted to diketogulonic acid. He has concluded that glutathione is not responsible for the reduction of dehydroascorbic acid in rabbit blood. He has shown, along with the reduction of dehydroascorbic acid to ascorbic acid, in the rat liver there is a fall in the glutathione level. He suggests that glutathione is responsible/

responsible for this reduction.

Dehydroascorbic acid bears a close resemblance in structure and properties to alloxan, and Patterson understood the significance. Patterson demonstrated the diabetogenic action of dehydroascorbic acid in albino rats when injected intravenously with large doses (60). Dehydroisoascorbic acid (61) and dehydroglucoascorbic acid were shown to be diabetogenic while diketogulonic acid was not (62). The diabetes following dehydroascorbic acid injections is permanent and may lead to cataracts in the albino rats (63). Patterson and Mastin (51) showed some effects on the central nervous system on injection of dehydroascorbic acid into albino rats. Patterson and Lazarow (64) demonstrated that glutathione, cysteine and 2:3-dimercapto propanol (BAL) administered intravenously immediately before the intravenous injection of dehydroascorbic acid in diabetogenic doses prevented the onset of diabetes. Similar prevention results from previous injection of atropine (65) although atropine injected into splenectomised rats did not have any preventive action. Bilateral adrenalectomy in DHA-induced diabetic rats ameliorates the condition (66).

Merlini (67) did not confirm the diabetogenic action of dehydroascorbic acid in rats. Princiotto (68) produced diabetes in fasting rabbits by intravenous injection of dehydroascorbic acid in four equal doses totalling 0.6 g/Kg. given at fifteen minute intervals, while Banerjee, Belavady and Mukherjee (69) failed to confirm the diabetogenic action of dehydroascorbic acid in rabbits. They reported that intravenous injection of dehydroascorbic acid in doses of 1 g/Kg. failed to produce persistent hyperglycaemia/

glycaemia or a diabetic type of glucose tolerance curve. Bhattacharya (59) confirmed the diabetogenic effect of dehydroascorbic acid in albino rats but not in rabbits.

To gain an insight into the metabolism of dehydroascorbic acid when administered intravenously into the albino rat in diabetogenic doses, work was undertaken and the observations made on the tissue levels of ascorbic acid following the injections are reported here.

EXPERIMENTAL

Materials. A. Ascorbic acid - Roche Products, Ltd.

Metaphosphoric Acid. Reagent grade, B.D.H.

2:6 dichlorophenol indophenol, B.D.H.

Sodium Citrate, A.R., B.D.H.

Trichloracetic Acid, A.R., B.D.H.

Norit Charcoal, acid washed before use.

2:4 dinitrophenylhydrazine, A.R., B.D.H.

Thiourea, Reagent grade, B.D.H.

Quinone, Commercial, B.D.H. Resublimed before use.

Nembutal (Pentobarbitone Sodium, B.P.) Abbot Laboratories, Ltd.

Spectrophotometer, Unicam SP 600.

B. Young healthy Wistar Glaxo albino rats of both sexes, within the range of body weight (varying) from 130 g. to 200 g. were used in the experiments. The animals were inbred in the laboratory and were maintained on rat cubes and water ad libitum. The composition of the rat cubes was as follows :-

Fine bran - 17.4%, ground maize - 17.4%, ground Sussex oats - 17.4%, ground barley - 17.4%, white fish meal - 4.8%, meat and bone meal - 9.6%, dried skimmed milk - 14%, dried yeast - 1.2%, cod liver - 0.4%, sodium chloride - 0.4%

Methods./

Methods.

The experiments consisted in the determinations of ascorbic acid and total ascorbic acid (ASA + DHA + DKA) in the tissues and blood, before and after the injections of dehydroascorbic acid.

Tissues were obtained by sacrifice of the animals by stunning or under nembutal anaesthesia. The abdomen was opened up and the animal was bled from the abdominal aorta as completely as possible, the blood being collected in a heparinised syringe. The tissues, in requisite amounts, were successively removed, freed from adherent tissue, wiped dry on a filter paper and immediately immersed in 5 - 10 volumes of aqueous solution of metaphosphoric acid or trichloracetic acid contained in previously weighed vessels. The aqueous solutions of 3% (w/v) metaphosphoric acid and 4% (w/v) trichloracetic acid were prepared fresh on the day of the experiment and used as protein precipitating and extracting agents for ascorbic acid and total ascorbic acid respectively. The process of removal of tissues was speedily completed and did not take longer than 10 minutes. The weights of the tissues were found by the difference to the nearest milligram by weighing on a chemical balance. The weights of the suprarenals was obtained by removing them from the extracting media. They were wiped dry and the weights to the nearest milligram were found on a torsion balance. Following this the tissues were ground with a pestle in a glass mortar under the respective extracting solution with acid washed white sand till they were completely pulped. More of the extracting agents were run in and the extracts removed to volumetric flasks. Repeated rinsings were made to ensure complete/

complete transfer of the ground tissue. Volumes were made up in such a way that the final concentration of ASA etc. in the extracts were near the middle of the calibration ranges (5 - 10 ɣ). The contents of the flasks were thoroughly shaken and centrifuged for 15 minutes at 2000 r.p.m. The clear supernatants were decanted off for further processing.

Estimations of ascorbic acid in the tissue extracts were made by the indophenol method described by Stewart, Horn and Robson (39). The principles on which the method is based are as follows:-

Ascorbic acid is the principal or perhaps the only substance, in acid extracts of tissues, that reduces the dye 2:6 dichlorophenolindophenol to a colourless compound at a pH of 1 to 4 within a period of 30 seconds. For the purposes of this extraction, metaphosphoric acid is the reagent of choice. The amount of ascorbic acid is proportional to the degree of reduction of the dye. Under the specified conditions, interference from other substances that reduce the dye, such as sulphydryl compounds etc., is minimum. In practice, an excess of the dye in aqueous solution, freshly made, is allowed to be reduced by a measured volume of the metaphosphoric acid extract of tissue and at the end of 30 seconds the degree of reduction is determined photometrically. With each experiment a calibration curve was run simultaneously. Appropriate standards containing 1,2,4,6 and 8 ɣ of ascorbic acid per ml. in metaphosphoric acid as used for the tissues, were prepared and carried through the entire procedure as the test solutions. The photometric densities were plotted and a calibration curve obtained.

During the course of the experiments certain anomalies in the results

were/

were noted. The values for total ascorbic acid determined were sometimes lower than the values of ascorbic acid obtained by the indophenol method. To find out whether sulphydryl compounds were interfering in these estimations, experiments were made with the p-chloromercuribenzoic acid method recently reported by Owen, Iggo and Horn (71). The trial experiments with chloromercuribenzoic acid were performed with the metaphosphoric acid extracts of tissues and the standard solutions of ascorbic acid prepared for use in the indophenol method. Along with these estimations in the indophenol method, estimations of total ascorbic acid by the Roe and Kuether method (vide infra) were done in two cases. Chloromercuribenzoic acid solution was prepared as directed and 1 ml. of this solution was added to 4 ml. of the metaphosphoric acid extract and processed. For a set of blanks, metaphosphoric acid extracts were also treated similarly with 0.05N. NaOH solution used for dissolving the chloromercuribenzoic acid. Standard curves with chloromercuribenzoic acid-treated standard solutions and alkali-treated standard solutions of ascorbic acid were run simultaneously with each experiment and values were read off the respective curves. The results of these experiments are presented in Table A.

From Table A it will be observed that in the case of the liver the values with chloromercuribenzoic acid tend to be variable, in the kidneys they tend to be lower and in the suprarenal and spleen they tend to be higher, within limits of experimental error. But the simultaneous estimations of total ascorbic acid with Roe and Kuether method gave values for the kidney which were lower than those obtained with chloromercuribenzoic acid treated metaphosphoric acid extract.

The/

Table A.

Concentrations of ascorbic acid in the tissues obtained before and after treatment of the tissue extracts with p-chloromercuribenzoic acid (CMB).

expressed as mg. per 100 g. tissue

Tissue	Indophenol method with unbuffered extract	Indophenol method with buffered extract	Extract treated with CMB	Extract treated with alkali (Control)	Total Ascorbic acid Roe & Kuether method
Liver	21.5		23.1		22.4
	19.8		20.5	21.8	19.2
	30.2	25.5	30.4	29.8	
	25.6	24.8	25.4	26.1	
Kidney	9.0		8.5		8.3
	9.5		8.8	11.2	8.7
	12.4	9.2	11.5	11.9	
	12.1	10.4	12.0	13.3	
Supra-renal	400.0		460.0		495.0
	460.0		500.0	560.0	447.2
	466.2	466.2	473.0	466.2	
Spleen	39.4	39.4	41.1	40.3	
	31.0	30.2	31.8	31.3	

The results were inconclusive so far as these anomalies in the ASA and TASA values were concerned. Mindlin and Butler (72) recommended a pH of 4.1 for the final reaction mixture with indophenol and metaphosphoric acid extract. Bessey (73) suggested a pH of 3.5 approximately for the reaction. Between pH 3 to 4 the indophenol fades least and the reaction becomes more specific. So test experiments with buffered extracts were made. In the case of the kidneys the values obtained with buffered extracts were almost always lower than those obtained with unbuffered extracts. For the purposes of the experiments, the metaphosphoric acid extracts were buffered to a pH of 3.5 to 4 with a fresh solution of sodium citrate of suitable concentration. A separate calibration curve with buffered solutions of ASA in metaphosphoric acid, in the range of concentrations mentioned above was run simultaneously with the test solutions on each day of experiment. The estimations for ascorbic acid in the tissues were completed within 6 hours after removal of the tissues every day.

Estimations of total ascorbic acid were made with the method described by Roe and Kuether (74). The principles underlying this method are as follows. Ascorbic acid is oxidised to dehydroascorbic acid with a mild oxidising agent. Dehydroascorbic acid undergoes spontaneous transformation into diketogulonic acid in mildly acid solution. When treated with 2:4-dinitrophenylhydrazine, DHA and DKA form a derivative, a bis-2:4-dinitrophenylhydrazone. The rate of coupling of 2:4-dinitrophenylhydrazine with DKA is faster than with DHA and possibly DHA is converted to DKA before coupling takes place. When the bis-2:4-dinitrophenylhydrazone so formed, is treated with 85% sulphuric acid,

a/

a highly stable reddish brown product is formed which is soluble in the acid. This solution absorbs maximally at about 540 mμ. The colour obtained in this way is measured photometrically, the intensity being proportional to the total ascorbic acid originally present. The coupling reaction is carried out in a medium containing reducing agents, e.g. thiourea, to avoid interference from non-ascorbic acid chromogens.

A standard curve was prepared on each day of the experiment in the following way. Appropriate standard solutions of ascorbic acid, in concentrations ranging from 0.625 to 5 γ per ml., were made up in 4 per cent (w/v) trichloracetic acid as used for the extraction. These were run, along with the test solutions, through the entire procedure. From the photometric densities a standard curve was obtained and the values for the test solutions were read off the curve.

The pancreas in the rat is a very diffuse gland intimately bound up with omentum and other tissues. Preliminary trials with estimations for ASA and TASA in two different extracting agents, namely metaphosphoric acid and trichloracetic acid, gave values which were widely different. Proper distribution of the true glandular tissue, in two different precipitants, from the same gland removed at dissection is almost an impossibility. To avoid this random distribution and the consequent errors, the gland, when removed, was always immediately put in metaphosphoric acid only, and extracted.

The choice of metaphosphoric acid as the extracting agent was made for the following reasons. Estimations of ascorbic acid in trichloracetic acid with indophenol are erroneous owing to the fact that a certain amount

of/

of oxidation of ascorbic acid takes place. The rate of fading of indophenol solution in the presence of trichloracetic acid is more rapid than that in the presence of metaphosphoric acid (39). Experiments with mixtures of metaphosphoric acid and trichloracetic acid showed that the mixture decolourised indophenol much more rapidly than did metaphosphoric acid alone as will be evident from Fig. 1.

Preliminary trials with the adoption of the mixture of metaphosphoric acid and trichloracetic acid, in place of trichloracetic acid, in the Roe and Kuether procedure showed that almost identical calibration curves and comparable values could be obtained as in the original method, as shown in Fig. II. Two sets of standard solutions of ascorbic acid in trichloracetic acid and in a mixture of metaphosphoric acid-trichloracetic acid were prepared and subjected to the same procedure simultaneously. The data obtained were plotted in the same figure. The slopes of the curves are almost identical. For the purposes of these experiments, 5 parts of 3% (w/v) metaphosphoric acid extract of pancreas were mixed with 3 parts of 6% (w/v) trichloracetic acid and the mixture was subjected to the analytical procedure of Roe and Kuether and run along with the other test solutions. Separate calibration curves were not made, the values being read off the curve for the original method.

The problem of the ascorbic acid values exceeding those of the total ascorbic acid, in some of the estimations, was considered from a different angle. Anatomical differences in the representative samples of tissues in two dissimilar extracting agents and the repeated weighings thereof may introduce errors in the estimated values. To avoid this source of error, estimat-

ions/

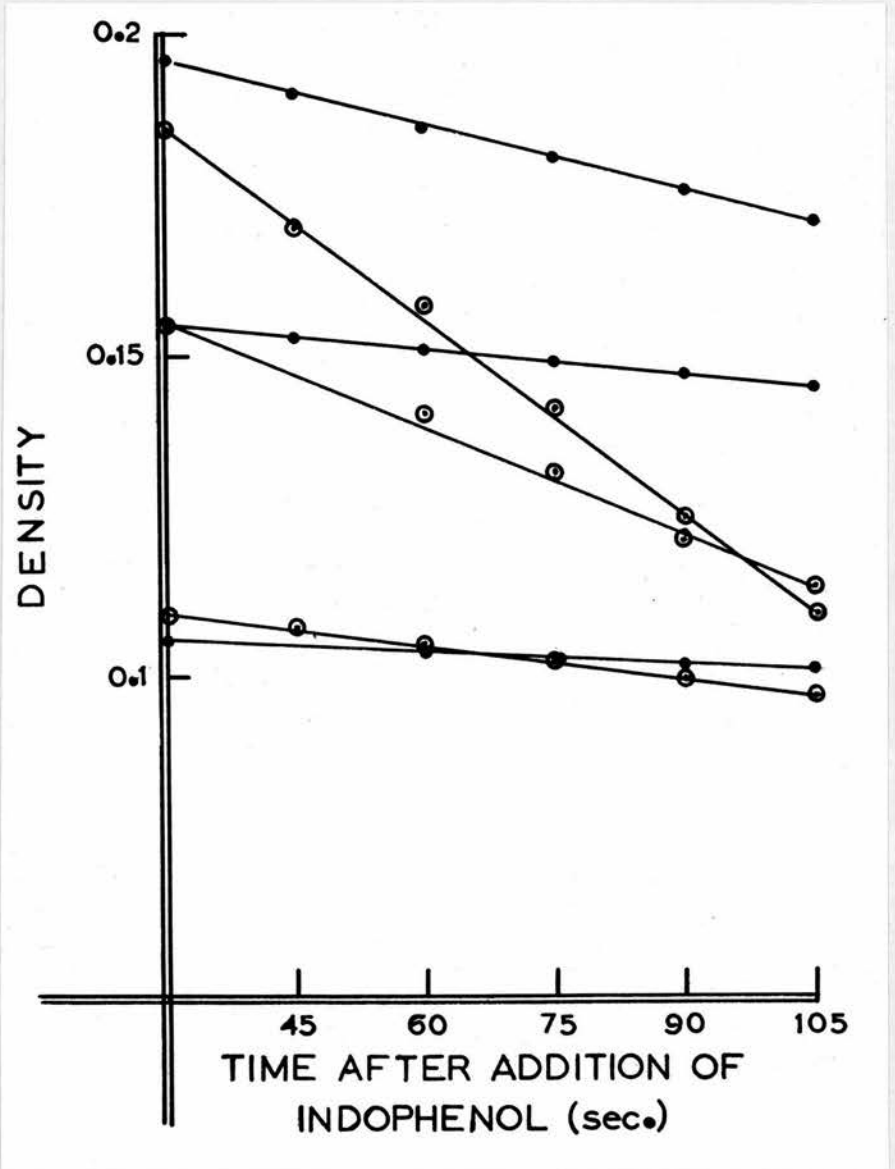


Fig. 1.

The effect of time on the density at 480 mμ. of 1.0 ml. of the indophenol solution (4 mg/100 ml.) in the presence of 2.0 ml. of standard ascorbic acid solutions (2, 4 and 6 γ per ml. from above downwards). Initial reading taken at 30 seconds. ●——●, standard ascorbic acid solutions in 3% (w/v) metaphosphoric acid; ⊙——⊙, standard ascorbic acid solutions in the mixture of 3% (w/v) metaphosphoric acid and 6% (w/v) trichloracetic acid.

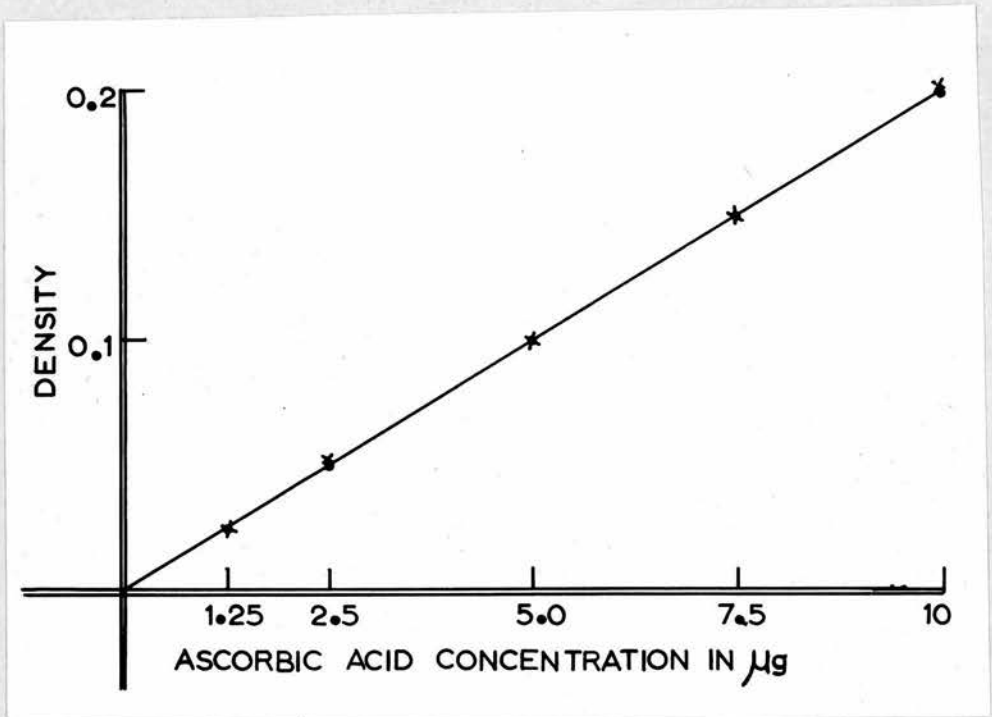


Fig. 2.

Standard curves obtained in the original and modified Roe and Kuether methods. The dots represent the densities obtained in the original method. The crosses represent the densities obtained by the use of the mixture of 3% (w/v) metaphosphoric acid and 6% (w/v) trichloracetic acid.

ions of total ascorbic acid were made in some cases with metaphosphoric acid extracts, prepared for the estimation of ascorbic acid. In these estimations the technique proposed by Bolin and Book (75) was followed. The principles on which the method is based are as follows. Quantitative oxidation of ascorbic acid in metaphosphoric acid extracts of tissues is brought about by the use of a strong solution of 2:6-dichlorophenol indophenol. The colour due to the excess of dye is destroyed by thiourea subsequently. The oxidised ascorbic acid is treated with 2:4-dinitrophenylhydrazine and a bis-2:4-dinitrophenylhydrazone is formed. On treatment with 85% sulphuric acid this gives a reddish brown colour which is measured photometrically at 540 mμ. The density of the colour produced is proportional to the total ascorbic acid originally present. Standard curves, for this method, were again prepared on each day of the experiment. Standard solutions of ascorbic acid in 3% (w/v) metaphosphoric acid, in the concentration range of 1 to 8 γ per ml., as made up for the estimation of ascorbic acid by the indophenol method, were used. These were treated in the same way as the test solutions.

Recovery Studies.

Recoveries of ascorbic acid added to the tissue extracts were made. The addition of ascorbic acid was done in two different ways. In the first group, 1 mg/100 ml. ascorbic acid was dissolved in 3% (w/v) metaphosphoric acid or 4% (w/v) trichloracetic acid and the tissues were extracted with these enriched media. In the other group 1 mg/100 ml. ascorbic acid was dissolved in the tissue extracts of metaphosphoric acid or trichloracetic acid. The results of recoveries from these samples are presented in Table B.

From/

Table B.

Recoveries of ascorbic acid added to the tissue extracts using the various methods.

Methods	Tissues			
	Liver	Kidney	Suprarenal	Spleen
ASA(A) without buffer	Mean 96.1% (95%,97.3%)	Mean 83% (78%, 88%)		
ASA(B) without buffer	Mean 98.3% (96%,99%,100%)	93%	99%	102%
ASA(A) with buffer	93%			
ASA(B) with buffer		99%	100%	
Total ASA(A) Roe & Kuether	Mean 96.1% (85%, 107%)			
Total ASA(B) Roe & Kuether	98.3%	96.5%		100%
Total ASA(A) Bolin & Book				
Total ASA(B) Bolin & Book	87%	99%	104.5%	

ASA(A) ... ascorbic acid added to the precipitating agent
ASA(B) ... ascorbic acid added to the tissue extract.

From Table B it will be noted that in the case of the liver, the recoveries of ascorbic acid added to the metaphosphoric acid before extracting the tissue were 93% and 96.1% (mean) respectively, using buffered and unbuffered extracts in the indophenol method. 98.3% (mean), of that added to the extract, was recovered using unbuffered test solutions. Recoveries as total ascorbic acid of the ascorbic acid added before extraction were 96% (mean) by the Roe and Kuether method, and, of that added after extracting, were 98.3% by the 'Roe and Kuether' method and 87% by the 'Bolin and Book' method.

In the case of the kidney, the recovery of ascorbic acid added to the extractant before precipitation was 85% (mean) as ascorbic acid using unbuffered samples in the method. Of that added after extraction 99% was recovered as ascorbic acid by using buffered test solutions in the method, and 96.5% and 99% as total ascorbic acid by the 'Roe and Kuether' and 'Bolin and Book' methods respectively.

Using extracts of the suprarenal glands, to which ascorbic acid was added, the recoveries in the indophenol method were 99% (without buffering the extract) and 100% (with buffered extract), while in the 'Bolin and Book' method it was 104.5%.

When extracts of the spleen were used, to which the ascorbic acid was added, 102% was recovered as ascorbic acid in unbuffered samples and 100% as total ascorbic acid in the Roe and Kuether method.

Experiments with dehydroascorbic acid :-

Observations were made on the tissue levels of ascorbic acid following the/

the intravenous injections of dehydroascorbic acid to the albino rats and as a preliminary to this study the levels of ascorbic acid in the tissues of the control animals of both sexes were obtained. These animals were not treated in any way and were sacrificed by stunning.

Patterson has produced hyperglycaemia and diabetes in the albino rats by using various dose levels of dehydroascorbic acid in his investigations. Following their investigations on sulphydryl protection against the diabetogenic action of dehydroascorbic acid, Patterson and Lazarow (64) reported that a preliminary desensitising dose of 0.2 g/Kg. followed by a dose of 0.7 g/Kg. body weight of dehydroascorbic acid given intravenously on three successive days produced diabetes in 87% of the albino rats. This dose level was selected for the purpose of this investigation and the following scheme was adopted for the study of the tissue levels of ascorbic acid.

Plan of the Experiments.

A Establishment of the control values of ascorbic acid.

B DHA injection experiments

(a) DHA injection (Detoxicating dose followed by main dose) ... removal of tissues after 15 minutes interval.

(b) DHA injection (Detoxicating dose and main dose) ... removal of tissues after an interval of 24 hrs.

(c) DHA injection (Detoxicating dose and main dose) on the first day.

Main dose of DHA repeated without detoxication on the/

the second day.

... removal of tissues after an interval of 24 hrs.

(d) DHA injection (Detoxicating dose and main dose) on the first day.

Main dose repeated on the second day.

Main dose repeated on the third day.

... removal of tissues after an interval of 15 minutes.

(e) DHA injection - same as in (d).

... removal of tissues after an interval of 8 days or more.

Dehydroascorbic acid was prepared according to the method described by Patterson (61). 1.08 g. of ascorbic acid was dissolved in 10 ml. of 0.9% sodium chloride solution and shaken for 15 minutes with an equal volume of an ethereal solution containing 0.66 g. of freshly sublimed quinone. The dehydroascorbic acid solution was separated and washed five times with an equal volume of ether. The excess ether was subsequently removed by suction under a filter pump. The solution, so prepared, was assumed to contain 100 mg/ml. of dehydroascorbic acid. The solution is stable for more than two hours at room temperature and is reducible (95 - 98 per cent) to ascorbic acid by H_2S at pH 3.5 and 37°C. It contains traces of hydroquinone as shown by a positive test with Chloramine T. The solution of dehydroascorbic acid was prepared fresh on each day that the injections were given and was used within one hour of preparation.

The injections of dehydroascorbic acid were given in the tail veins of/

of the rats, after dilatation in warm water, with an all-glass syringe and a 26 gauge needle. Prior to the injection the animals were starved for 12 to 24 hours. A preliminary desensitising dose of about 0.2 g/Kg. body weight of DHA was given and within 10 to 30 minutes the main dose of 0.7 g/Kg. DHA was administered. The main dose was repeated on the second and third day approximately at 24 hour intervals, when necessary without further desensitising. In the acute experiments, the animals were bled from the 10th to 14th minute and the tissues were successively removed from the 15th to 21st minute after the injection of the main dose and within 30 - 35 minutes after the initial desensitising dose.

In order to facilitate accurate timing of the removal of tissues and the collection of blood from the abdominal aorta the DHA-injected animals were sacrificed under nembutal anaesthesia. 6 mg/100 g. body weight of nembutal was administered intraperitoneally for this purpose. In the acute experiments with DHA (in groups (a) and (d) of the plan) the injection of nembutal preceded that of the main dose of DHA. Control values of tissue ascorbic acid under nembutal anaesthesia were also obtained.

As the solution of dehydorascorbic acid contains traces of hydroquinone, it was necessary to find out whether any change in the values of ascorbic acid in the tissues or interference in the methods were being caused by the use of the above solution. To simulate conditions control animals were injected with a solution of hydroquinone prepared as follows. 0.66 g. of hydroquinone was dissolved in 10 ml. of ether and shaken with 10 ml. of 0.9% sodium chloride solution for fifteen minutes, as in the method/

method of preparation of DHA. The saline solution was separated and extracted five times with pure ether, the excess of ether was removed by suction under a filter pump. The solution so obtained was used for intravenous injection without further treatment. The solution was injected in the same way as DHA on three successive days into control female rats. No apparent interference or significant change in the values of ascorbic acid was noted, so this was not repeated in the male control rats.

Estimations of blood sugar levels were done by the method of Hagedorn and Jensen (76). The total ascorbic acid concentration of blood was determined by the Roe and Kuether procedure (74). Soon after withdrawal, the blood was precipitated in 6% (w/v) trichloracetic acid solution and processed along with the tissue extracts in the above method.

Certain general observations on morbidity and mortality following the injections of dehydroascorbic acid are noted. The phenomena of "tachyphylaxis" were noted with varying degrees of severity in all the animals. After recovery from the immediate effects of the administration of DHA on the first day the animals, generally, did not take any food and varying losses of weight were observed. After the second dose weakness, dehydration and loss of weight became marked features. Some of the animals developed dysenteric symptoms. The loss of weight in a group of 23 animals averaged 26.79 g. ± 4.5 g., while in another group of 8 animals, weight loss did not take place. These animals were not apparently ill. Following the third injection, the weight loss and dehydration progressively increased and the animals passed into a critical phase of illness for the next 2 - 4 days. The greatest mortality in the animals/

animals occurred during this period in this series. Hyperglycaemia was present in some of the animals (present in 4 out of 5 tested). On recovery from this critical phase the animals gradually regained weight and appeared apparently normal.

RESULTS

The data obtained in the different experiments are presented in the following pages. Tables 1 to 3 and 5 to 7 summarise the results obtained in the different control groups of animals used in this investigation. The results obtained following the intravenous injections of DHA are summarised in Tables 8 to 17. For descriptive purposes, the values obtained by the indophenol methods are spoken of as "ASA values" while those obtained by the Roe and Kuether method and the Bolin and Book method are designated "TASA values".

Tables 1 and 2 summarise the ASA and TASA concentrations in the tissues and organs of the control animals of both sexes. These animals were not subjected to any injection and were sacrificed by stunning. The ASA and TASA concentrations in the liver, kidney, suprarenal, pancreas and spleen of six male rats with a mean body weight of 163 g. (range 155 to 170 g.) are presented in Table 1.

It will be observed that the ASA values obtained by the indophenol methods, using buffered and unbuffered tissue extracts, are not significantly different. Thus, using buffered extracts, the liver of the male rat was found to contain 30.2 ± 4.89 mg.ASA/100 g., the corresponding figure for unbuffered solutions being 30.0 ± 4.15 mg.ASA/100 g. In the case of the kidneys the figures for buffered and unbuffered solutions were 11.8 ± 1.89 mg/100 g. and 12.6 ± 1.56 mg/100 g.; for the suprarenals/

Table 1.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the control male rats sacrificed by stunning.

No.	Weight in g.	Tissue	Indophenol method without buffered extract mgASA/100g.	Indophenol method with buffered extract mgASA/100g.	Bolin & Book method mgTASA/100g.	Roe & Kuether method mgTASA/100 g.
1	165	Liver	32.9	33.3	37.0	33.7
2	163	"	32.5	30.4	30.4	29.1
3	165	"	33.8	37.4	35.6	37.4
4	170	"	26.3	25.9	26.3	25.0
5	160	"	31.0	30.5	33.0	34.1
6	155	"	23.5	23.9	28.1	32.0
Mean	163	Mean	30.0 ± 4.15	30.2 ± 4.89	31.7 ± 4.21	31.9 ± 4.33
1		Kidney	11.6	11.2	13.3	14.0
2		"	11.5	10.8	13.2	13.5
3		"	15.1	15.0	15.7	16.3
4		"	14.0	13.0	15.5	15.7
5		"	11.7	10.0	13.6	13.1
6		"	11.6	10.5	12.7	13.7
		Mean	12.6 ± 1.56	11.8 ± 1.89	14.0 ± 1.27	14.4 ± 1.30
1		Supra-	287.5	350.0	350.0	312.5
2		renals	484.6	469.2	515.4	446.8
3		"	447.1	482.4	511.8	514.2
4		"	421.1	436.8	418.4	355.5
5		"	414.7	297.1	341.2	287.5
6		"	453.3	466.7	433.3	446.2
		Mean	418.1 ± 68.65	417.0 ± 75.80	428.4 ± 75.36	393.8 ± 88.89
1		Pancreas	11.0	11.6	13.8	12.9
2		"	11.8	11.5	12.1	12.1
3		"	14.4	15.3	13.2	14.3
4		"	11.6	11.6	12.3	11.6
5		"	11.6	11.4	12.8	12.0
6		"	13.4	13.3	14.7	13.2
		Mean	12.3 ± 1.31	12.5 ± 1.57	13.2 ± 0.98	12.7 ± 0.99
2		Spleen	29.5	26.9	35.5	40.7
3		"	30.7	34.9	36.1	47.6
4		"	33.6	34.1	39.8	42.8
5		"	15.7	20.8	30.1	38.7
6		"	26.9	26.2	34.6	39.5
		Mean	27.3 ± 6.91	28.6 ± 5.90	35.2 ± 3.48	41.9 ± 3.56

renals they were 417.0 ± 75.80 mg/100 g. and 418.1 ± 68.65 mg/100 g.; for the pancreas they were 12.5 ± 1.57 mg/100 g. and 12.3 ± 1.31 mg/100 g.; and for the spleen they were 28.6 ± 5.90 mg/100 g. and 27.3 ± 6.91 mg/100 g. respectively. The TASA values obtained by the two phenylhydrazine methods, in the liver, kidney and pancreas, are very close. The liver contained 31.9 ± 4.33 mg.TASA/100 g. by the Roe and Kuether method and 31.7 ± 4.21 mg.TASA/100 g. by the Bolin and Book method. The kidney contained 14.4 ± 1.30 mg/100 g. by the first method, the corresponding figure in the second method being 14.0 ± 1.27 mg/100 g.; while in the pancreas the figures were 12.7 ± 0.99 mg/100 g. and 13.2 ± 0.98 mg/100 g. respectively. A comparison of the ASA values and TASA values in the liver, kidney and pancreas shows that there is very little difference between the two values, though the TASA values are slightly higher. It will be seen in some of the estimations, that the ASA value has exceeded the TASA value. The differences, observed here and in other experiments to be described later, are not explicable on the basis of experimental errors alone. It seems that some, but not all tissues must contain significant quantities of material which interferes with the analytical methods employed. The exact cause of this anomaly could not, however, be ascertained. In the suprarenals, the TASA value obtained by the Roe and Kuether method, namely 393.8 ± 88.89 mg. TASA/100 g. is lower than the ASA values (417.0 ± 75.80 mg/100 g. and 418.1 ± 68.65 mg/100 g.) and the TASA value of 428.4 ± 75.36 mg/100 g. obtained by the Bolin and Book method/

Table 2.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the control female rats, sacrificed by stunning.

Body weight g.	Tissue	Indophenol method without buffer mgASA/100g.	Indophenol method with buffer mgASA/100g.	Bolin & Book method mgTASA/100g.	Roe & Kuether method mgTASA/100g.
150	Liver	21.3			21.2
140		31.7			27.7
178		23.8			26.3
175		21.0			22.0
180		20.0			23.7
190		19.9			22.3
160		16.5	16.2	18.8	23.2
155		17.7	16.3	23.1	24.6
Mean 166	Mean	21.5± 4.69			Mean 23.9±2.21
	Kidney	14.5			9.3
		16.1			13.8
		13.2			9.8
		10.9			10.4
		11.6			13.2
		12.8			10.0
		7.4	4.9	9.3	9.5
		9.5	5.5	8.9	8.7
	Mean	12.0 ± 2.78			10.6 ± 1.87
	Supra-renal	413.6			394.1
		400.0			407.7
		427.8			366.0
		380.9	342.9	404.8	369.5
		352.2	387.0	434.8	365.5
	Mean	394.9			Mean 380.6
	Spleen	24.4			29.3
		16.3			34.4
		25.3			34.0
		24.5			32.9
		18.5	17.1	35.4	37.7
	Mean	21.8			Mean 32.4
	Pancreas	12.9			8.8
		8.7			5.9
		6.5			6.7
	Mean	9.4			Mean 7.1
	Brain	17.7			45.0
	Muscle	4.8			3.2

method. In the case of the spleen, the TASA value of 41.9 ± 3.56 mg/100 g. obtained by the Roe and Kuether method is higher than 35.2 ± 3.48 mg/100 g. obtained by the Bolin and Book method.

In Table 2, the results obtained in 8 female rats with a mean body weight of 166 g. (range 140 to 190 g.) are shown. The tissues examined were the liver, kidney, suprarenals, spleen, pancreas, brain and voluntary muscle. Most of these data were obtained at the preliminary stages of the investigation and the anomaly, referred to above, is more apparent here and as such had led to the adoption of the different methods of estimation. In the liver the ASA value by the indophenol method using unbuffered extracts was 21.5 ± 4.69 mg/100 g. while the TASA value by the Roe and Kuether procedure was 23.9 ± 2.21 mg/100 g. In the suprarenals, the corresponding figures for ASA and TASA were 394.9 mg./100 g. and 380.6 mg/100 g. respectively. It will be noted that the TASA values in the cases of the kidney of 10.6 ± 1.87 mg/100 g., pancreas of 7.1 mg/100 g. and muscle of 3.2 mg/100 g. are all lower than the corresponding ASA values in the kidney of 12.0 ± 2.78 mg/100 g., pancreas of 9.4 mg/100 g. and muscle of 4.2 mg/100 g. The TASA values in the spleen and brain were 32.4 mg/100 g. and 45.0 mg/100 g. respectively and these are higher than the corresponding ASA values of 21.8 mg/100 g. in the spleen and 17.7 mg/100 g. in the brain.

On comparison of the ascorbic acid contents of the organs in the two sexes, it will be observed that the male rats have a higher ascorbic acid/

Table 3.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of adult male rats, sacrificed by stunning.

Weight in g.	Tissue	Indophenol method method without buffered extract mg.ASA/100 g.	Roe & Kuether method mg.TASA/100 g.
235	Liver	21.5	22.4
260		20.1	19.1
315		23.4	23.2
315		20.1	27.5
		Mean 21.3	23.1
	Kidney	8.9	8.3
		9.5	8.6
		9.2	11.7
		6.5	11.6
		Mean 8.5	10.1
	Suprarenal	400.0	495.0
		530.7	466.0
		419.5	480.0
		425.0	450.0
		Mean 443.8	472.8

Table 4.

The distribution of ascorbic acid in the tissues of the albino rat from literature

in milligrams per 100 g. of tissue.

(Figures for control values, converted to 100g. in some cases).

No.	Weight	Sex	Liver	Kidney	Supra-renal	Spleen	Brain	Muscle	Pancreas	Blood or plasma mg/100ml.	Method of estimation	Reference
1	75 days old		27.0	17.0			36.0	6.0			Indophenol	(77)
	1 year old		17.0	14.0			37.0	4.0			"	
2	220 - 230g.		19.0-24.0		218.0-460.0						Indophenol	(78)
3	Normal adult		26.6 (16.2-35.3)	14.4 (10.5-19.8)	337.0 (152.0-447.0)			6.2 (4.2-8.5)				(79)
4		Same sex	25.0,22.0 26.0,19.0	16.0,14.0, 16.0,10.0	361.0,368.0 395.0,294.0						Indophenol	(80)
5	85-150 g.	male	18.2±0.91		398±17		40.6±0.96			(Pl) 0.81±0.105	Indophenol	(81)
	240-260 g.	"	24.0±1.10		403±15.2		39.6±0.72			(Pl) 0.51±0.058		
6	250-450 g.	Male								(WB) 0.72±0.017	Roe & Kuether	
										(Pl) 0.87±0.028	Indophenol	(85)
	200-350 g.	female								(WB) 0.35±0.011	Roe & Kuether	
										(Pl) 0.33±0.019	Indophenol	
7	200-250 g.	male			362.6±20.4	37.0±1.9					Roe & Kuether	(82)
8	80-180 g.	male			514± 22						Roe & Kuether	(83)
9	138-208 g.	F(LE)	19±2	8.9±0.4	330±30	33±3		3.2±0.3		(WB) 0.81±0.09	Roe & Kuether	(84)
	94-162 g.	F(W)	19±1	8.2±0.5	380±20	38±1		2.2±0.1		(WB) 0.58±0.04		
	163 (155-170)	male	31.9±4.33	14.4±1.30	393.8±88.89	41.9±3.56			12.7±0.99		Roe & Kuether	Present author
	166 (140-190)	female	23.9±2.21	10.6±1.87	380.6	32.4		3.2	7.1			

concentration in the liver, kidneys, pancreas and the spleen. During the course of the study, it was found that the male rats, in the range of body weight used in this investigation, generally gained weight more rapidly than the female rats. To settle this point whether different rates of growth were responsible for the differences in ascorbic acid concentrations, analyses were made of the organs of male rats of high body weights and the results obtained are summarised in Table 3. These animals were found to contain 21.3 mg. as ASA and 23.1 mg. as TASA per 100 g. in the liver, 8.5 mg. as ASA and 10.1 mg. as TASA in the kidney and 443.8 mg. ASA and 472.8 mg. TASA in the suprarenals; these values agree well with the corresponding values in the female rats in Table 2.

Table 4 has been compiled from the literature and shows the concentrations of ascorbic acid reported in the organs and blood of the albino rats. These animals were used as control animals in the various investigations. The relevant details of sex, age and weight, where available, have been mentioned together with the method of estimation used. The Table requires no detailed comment. In general it shows that the concentrations of ASA previously reported in various rat tissues are of the same order of magnitude as those found in the present study.

The description of the results in the following pages is mainly based on the ASA values obtained by the indophenol method using buffered tissue extracts and TASA values obtained by the Roe and Kuether method. The variations from this practice are duly pointed out.

The/

The effects on the tissue concentrations of ascorbic acid following nembutal anaesthesia are summarised in Tables 5 and 6. These animals were sacrificed within 10 to 25 minutes after the administration of nembutal. It will be observed, that in the male rats in Table 5, the liver was found to contain 32.6 ± 2.57 mg.ASA/100 g. and 35.2 ± 2.63 mg.TASA/100 g. The kidneys contained 15.6 ± 2.94 mg.ASA/100 g. and 21.1 ± 4.59 mg.TASA/100 g. These values appear to be slightly higher than those in the previous control group but the differences are not statistically significant. In the female rats in Table 6, the liver contained 19.5 ± 3.22 mg.ASA/100 g. and 22.9 ± 3.29 mg.TASA/100 g., the figures for the kidney being 9.6 ± 1.43 mg.ASA and 12.5 ± 2.43 mg.TASA per 100 g. These values appear to be similar to those in the previous control group. It will be noted, however, that the TASA value in the kidney has increased and the anomaly described before is less apparent. In the male rats, the values for the suprarenals were 362.0 ± 72.65 mg.ASA and 368.6 ± 36.94 mg.TASA per 100 g. (figures slightly but not significantly lower than in the unanaesthetised animals), while the corresponding figures in the female were 272.0 ± 72.22 and 273.3 ± 68.00 per 100 g. (these figures appear to be definitely low when compared with the normal levels). In the pancreas, in both groups, and in the spleen and brain in the female rat the ascorbic acid concentrations are well within the normal levels. Reference will be made to these minor variations in due course.

The results obtained following the intravenous injection of an ether-extracted solution of hydroquinone to female rats on three successive days and/

Table 5.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the control male rats, sacrificed under nembutal anaesthesia.

No.	Weight in g.	Tissue	Indophenol method without buffered extract mgASA/ 100g.	Indophenol method with buffered extract mgASA/ 100g.	Bolin & Book method mgTASA/ 100g.	Roe & Kuether method mgTASA/ 100g.	Whole Blood "R & K" TASA mg/100ml.
1	160	Liver	29.7	29.8	32.7	35.5	
2	150		35.5	35.5	32.6	37.0	
3	160		33.2	32.1	33.2	33.1	
4	170		33.6	34.2	33.9	34.9	
5	155		35.5	34.7	37.5	39.1	
6	160		29.9	29.4	27.9	31.8	0.37
Mean	159	Mean	32.9 ± 2.58	32.6±2.57	33.0±3.07	35.2±2.63	
		Kidney	14.9	13.5	18.5	19.1	
			21.9	20.1	24.0	25.5	
			17.8	16.7	19.0	21.8	
			17.3	16.5	20.3	22.3	
			18.5	15.5	21.0	24.9	
			13.2	11.5	12.1	13.0	
		Mean	17.3±3.01	15.6±2.94	19.2±3.96	21.1±4.59	
1		Adrenal	335.7	357.1	385.7	388.5	
3			478.6	485.7	471.4	420.0	
4			280.0	340.0	320.0	334.4	
5			338.5	296.2	323.1	366.7	
6			346.2	330.8	330.8	333.3	
		Mean	355.8	362.0	366.2	368.6	
			±73.52	±72.65	±64.60	±36.94	
1		Pancreas	8.6	8.7	10.3	9.4	
3			11.3	8.6	9.2	8.2	
4			11.6	13.3	13.9	14.1	
5			11.9	9.2	10.0	11.0	
6			9.0	7.2	7.5	6.6	
		Mean	10.5±2.30	9.4±2.29	10.2±2.34	9.9±2.86	

Table 6.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the control female rats, sacrificed under nembutal anaesthesia.

No.	Weight in g.	Tissue	Indophenol method without buffered extract mgASA/100g.	Indophenol method with buffered extr. mgASA/100g.	Bolin & Book method mgTASA/100g.	Roe & Kuether method mgTASA/100g.	Whole Blood (R & K) TASA mg/100ml.
1	160	Liver	22.6	19.1	23.8	22.1	
2	170		21.4	19.1	22.3	22.8	
3	160		17.6	16.0	21.2	20.2	0.3
4	160		18.7	17.5	20.0	19.5	
5	158		25.1	25.4	27.5	28.6	
6	165		23.1	20.1	-	24.3	
		Mean	21.4±2.82	19.5±3.22	23.0±2.90	22.9±3.29	
		Kidney	12.1	8.7	14.5	13.8	
			13.1	10.1	13.8	15.0	
			10.6	7.2	9.5	8.8	
			13.2	11.2	12.3	11.2	
			13.6	10.5	14.6	14.7	
			12.6	9.8	14.5	11.3	
		Mean	12.5±1.06	9.6±1.43	13.2±2.01	12.5±2.43	
		Adrenal	209.3	200.0	174.1	245.2	
			254.2	254.2	250.0	206.3	
			255.0	310.0	290.0	229.2	
			390.5	392.9	419.1	381.8	
			209.5	204.8	228.6	245.7	
			255.0	270.0	365.0	331.3	
		Mean	262.3±66.64	272.0±72.22	281.1±85.12	273.3±68.00	
		Pancreas	6.4	5.5	7.5	7.0	
			8.4	9.1	8.9	9.5	
			5.9	-	6.6	5.3	
			8.5	7.9	7.4	6.6	
			8.0	7.1	8.9	9.2	
		Mean	7.4±1.21	7.4	7.9±1.01	7.5±1.79	
		Spleen	11.9	10.1	27.3	30.2	
			24.4	22.5	39.5	-	
			17.2	18.0	31.9	38.2	
			14.4	13.1	31.8	33.1	
		Mean	17.0	16.0	32.6	33.8	
		Brain	47.5	46.7	53.0	45.8	

Table 7.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the control female rats, injected with hydroquinone solution.

No.	Weight in g.	Tissue	Indophenol method without buffered extract mgASA/100 g.	Roe & Kuether method mgTASA/100g.	Blood Sugar mg/100 ml.
1	155	Liver	22.2	25.0	124
2	150		19.0	24.0	114
4	160		25.1	30.2	110
6	145		24.2	28.6	113
			Mean . . 22.6	27.0	
1		Kidney	16.0	16.1	
4			17.6	15.4	
6			15.8	16.4	
			Mean . . 16.5	16.0	
		Supra-renal	282.0	314.2	From rats 1,2,
			292.3	320.0	" " 4,5,6
			Mean . .287.2	317.1	
		Muscle	3.3	3.5	

and sacrificed under nembutal anaesthesia, are summarised in Table 7. The tissues examined were the liver, kidney, suprarenals and voluntary muscle. In these experiments, the indophenol method, using unbuffered extracts, was used for the ASA estimations while the TASA concentrations were estimated by the Roe and Kuether procedure. The suprarenal glands from three animals were pooled for analysis, in these experiments. It will be observed that in the liver there was 22.6 mg.ASA/100 g. and 27.0 mg.TASA/100 g. and these correspond closely to the control values. In the kidney the ASA concentration was 16.5 mg./100 g. and the TASA concentration was 16.0 mg/100 g.; these values are slightly higher than the controls. The suprarenal glands were found to contain 287.2 mg.ASA/100 g. and 317.1 mg.TASA/100 g. These values are slightly below the controls. These minor variations do not materially affect the results of this investigation.

The results summarised in Tables 8 and 9 show the immediate effects on the concentrations following the intravenous administration of a single dose of 0.7 g/Kg. body weight of DHA to the male and female rats. It will be noted that the greatest concentration of TASA has taken place in the kidneys, the figures being 487.6 mg/100 g. in the male and 693.4 mg/100 g. in the female rats. The concentration of TASA has increased in the other organs in the following order namely liver, pancreas and spleen. The liver was found to contain 308.2 mg.TASA/100 g. in the male and 394.4 mg./100 g. in the female. These values represent a 10 to 16 fold increase above the original levels. Of this amount in the liver in both sexes, about 82 per cent was present/

Table 8.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the male rats injected with dehydroascorbic acid (0.2g. and 0.7g. per Kg. body weight approx.). Tissues removed after 15 minutes from the completion of the injection.

No.	Weight in g.	Tissue	Indophenol method without buffered extract mgASA/100g.	Indophenol method with buff-extract mgASA/100g.	Bolin & Book method mgTASA/ 100g.	Roe & Kuether method mgTASA/ 100g.	Whole Blood (R & K)* mgTASA/ 100ml.
1	160	Liver	231.7	242.2	260.6	240.4	100.8
2	165		227.6	246.1	264.6	302.0	81.0
3	165		259.5	269.6	334.5	382.1	106.0
		Mean..	239.6 . . .	252.6 . . .	286.6 . .	308.2	
					Reduction	82%	
1		Kidney	287.2	286.5	483.4	501.0	
2			270.0	271.5	461.9	483.6	
3			276.9	283.4	443.7	478.3	
		Mean	278.0 . . .	280.5 . . .	463.0 . .	487.6	
					Reduction	57%	
1		Supra-	283.3	216.7	291.7	225.0	
2		renal	250.0	240.0	273.3	234.2	
3			253.3	273.3	273.3	256.7	
		Mean	262.2 . . .	243.3 . . .	279.4 . .	238.6	
		Pancreas	47.5	48.3	73.1	69.9	
			32.5	32.1	57.0	61.2	
			57.8	56.6	119.9	121.2	
		Mean	45.9 . . .	45.7 . . .	83.3 . .	84.1	
					Reduction	54%	
		Spleen	155.1	158.9	183.1	160.6	
			79.1	84.6	109.1	117.9	
			91.3	89.8	114.4	114.0	
		Mean	108.5 . . .	111.1 . . .	135.5 ..	130.8	
					Reduction	82%	

*Roe & Kuether method.

Table 9.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of female rats injected with dehydroascorbic acid (0.2g. and 0.7g. per Kg. body weight). Tissues removed after 15 minutes from the completion of injection.

Body weight Initial g.	Body weight Final g.	Tissue	Indophenol method without buffered extract mgASA/100g.	Indophenol method with buffer extract mgASA/100g.	Bolin & Book method mgTASA/100g.	Roe & Kuether method mgTASA/100g.	Whole Blood (R & K)* mgTASA/100ml.
160	150	Liver	317.5	320.6	336.0	417.6	105.5
160	148		313.7	306.5	344.8	369.1	90.5
163	152		363.6	352.8	370.1	396.5	114.0
		Mean	331.6	326.6	350.3	394.4	
				Reduction		83%	
		Kidney	336.0	351.7	643.5	655.7	
			321.2	307.4	664.6	680.1	
			405.2	378.0	730.6	744.5	
		Mean	354.1	345.7	679.6	693.4	
				Reduction		50%	
		Supra-renal	266.7	225.0	300.0	302.2	
			309.6	294.2	321.2	316.7	
			334.8	326.1	373.9	311.9	
		Mean	303.7	281.8	331.7	310.3	
		Pancreas	42.3	36.8	71.8	64.2	
			44.6	42.4	69.5	62.7	
			51.4	47.5	87.3	84.6	
		Mean	46.1	42.2	76.2	70.5	
				Reduction		59%	
		Spleen	65.3	65.3	88.0	98.1	
			70.1	67.5	100.3	111.6	
			60.1	49.7	75.4	-	
		Mean	65.2	60.8	87.9	104.9	
				Reduction		58%	

*Roe & Kuether method.

present as reduced ASA, the values being 252.6 mg.ASA/100 g. and 326.6 mg./100 g. in the male and female rats respectively. In the kidneys, the concentration of TASA has increased approximately 32 times the normal level in the male while the concentration in the female rat is about 63 times the original level. Of this quantity, only about 50 per cent was present as reduced ASA, the values being 280.5 mg.ASA/100 g. in the male and 345.7 mg.ASA/100 g. in the female. The concentration of TASA in the pancreas has risen about sevenfold in the male, the value being 84.1 mg.TASA/100 g., while in the female, the value of 70.5 mg.TASA/100 g. represents a tenfold rise approximately. Of this amount in the pancreas, about 50 per cent was found as reduced ASA. The male pancreas was found to contain 45.7 mg.ASA/100 g. while the female pancreas contained 42.2 mg.ASA/100 g. In the spleen, there has taken place a fourfold rise in TASA approximately. In the male, of the TASA of 130.8 mg./100 g. about 82 per cent, that is 111.1 mg./100 g. was present as reduced ASA, while the corresponding figures for spleen in the female of TASA and ASA were 104.9 mg.TASA/100 g. and 60.8 mg.ASA/100 g. respectively, which denotes about 58 per cent reduction. The concentration of TASA in blood under the same conditions was in the neighbourhood of 100 mg./100 ml. The suprarenals of the male rats were found to contain 243.3 mg.ASA and 238.6 mg.TASA per 100 g., the corresponding figures for the female rats being 281.8 mg.ASA and 310.3 mg.TASA per 100 g. Compared with the normals, which were 417.0 ± 75.80 mg.ASA/100 g. and 393.8 ± 88.89 mg.TASA/100 g. in the male rat and 394.9 mg.ASA/100 g. and 380.6 mg.TASA/100 g./

100 g. in the female rat, the concentrations of ASA and TASA in the suprarenals, instead of showing an increase following the injection, seem to have decreased. These figures indicate the extreme rapidity with which the injected DHA is reduced to ASA; in 30 minutes, over three quarters of an enormous dose of DHA has been reduced. The kidney is outstanding in containing the greatest proportion of DHA; it seems possible that the renal tissue may, like the leucocytes, tend to oxidise ASA rather than to reduce DHA. This possibility which might help to explain certain anomalies in the excretion of ASA deserves further investigation. Another point which deserves special notice is the decrease in the ASA concentration of the adrenals. This may indicate a stress reaction sufficiently powerful to bring about a disappearance of ASA faster than it can be replenished even from the very high concentrations in the circulating plasma.

Following the administration of a single dose of DHA, when the animals were sacrificed after an interval of 24 hours, the concentrations of ascorbic acid in the organs had changed and these results are summarised in Tables 10 and 11. It will be observed that in liver, kidney, spleen and brain, the concentrations are higher than normals in both sexes. Thus, the liver of male rats was found to contain 41.6 ± 2.78 mg.ASA/100 g. and 47.6 ± 4.46 mg.TASA/100 g. In the case of the kidney the figures were 23.3 ± 5.59 mg.ASA/100 g. and 30.1 ± 6.33 mg.TASA/100 g.; for spleen they were 27.4 mg.ASA/100 g. and 49.9 mg.TASA/100 g. and brain contained 51.3 mg.ASA/100 g. and 61.2 mg.TASA/100 g. In the female rats, the liver contained/

Table 10.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the male rats injected once with dehydroascorbic acid (0.2g. and 0.7g. per Kg. body weight). Tissues removed after an interval of 24 hours.

Weight in g. Initial	Final	Tissue	Indophenol method without buffered extract mgASA/100g.	Indophenol method with buffered extract mgASA/100g.	Bolin & Book method mgTASA/ 100g.	Roe & Kuether method mgTASA/ 100g.	Whole Blood mgTASA/ 100ml.
168		Liver	45.7	44.7	43.7	44.1	
150	140		41.0	38.4	41.7	50.9	
150	146		39.5	37.9	41.4	42.5	1.91
150	142		43.5	42.6	45.2	51.8	
150	144		44.2	42.8	39.7	44.2	
160	145		46.7	43.2	44.0	52.2	1.5
		Mean	43.4±2.75	41.6±2.78	42.6±2.03	47.6±4.46	1.705
		Kidney	31.1	29.8	32.4	41.7	
			19.2	16.8	25.5	28.6	
			27.5	29.6	30.9	32.7	
			21.6	17.7	25.1	25.1	
			25.4	22.5	20.8	25.6	
			26.9	23.1	25.7	26.7	
		Mean	25.3±4.29	23.3±5.59	26.7±3.87	30.1±6.33	
		Supra-renal	500.0	453.8	515.4	468.8	
			392.0	373.3	400.0	408.3	
			379.4	358.8	364.7	325.0	
			357.1	346.4	357.1	305.3	
			367.7	376.5	376.5	370.0	
			333.3	283.3	320.0	-	
		Mean	388.3±58.30	365.4±55.04	389.0±67.27	375.5±65.75	
		Pancreas	13.7	10.8	12.4	10.9	
			10.8	10.1	10.8	10.5	
			14.5	13.1	14.9	15.2	
			8.7	8.5	9.4	9.1	
			15.5	14.0	14.0	15.2	
			14.9	12.9	13.2	13.8	
		Mean	13.0±2.45	11.6±2.11	12.5±1.88	12.5±2.62	
		Spleen	20.8	20.6	36.0	42.3	
			35.2	35.2	51.9	55.8	
			24.6	24.4	42.9	51.1	
			29.5	29.5	42.1	50.4	
		Mean	27.5	27.4	43.2	49.9	
		Brain	57.9	51.3	55.7	61.2	

Table 11.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the female rats injected once with dehydroascorbic acid (0.2g. and 0.7g. per Kg. body weight). Tissues removed after an interval of 24 hours.

Body Weight Initial g.	Body Weight Final g.	Tissue	Indophenol method without buffered extract mgASA/100g.	Indophenol method with buffered extract mgASA/100g.	Bolin & Book method mgTASA/ 100g.	Roe & Kuether method mgTASA 100g.	Whole Blood R & K method mgTASA/ 100ml.
150	135	Liver	46.3	45.3	48.0	47.3	1.48
157	140		45.3	46.1	41.0	51.1	1.98
155	145		35.9	32.6	35.3	38.6	
160	148		42.4	41.4	41.2	40.8	
160	146		42.7	40.9	41.4	42.1	
162	143		44.0	41.8	43.3	45.3	
155	138		47.2	47.0	47.7	48.1	
		Mean..	43.4±3.75	42.2±4.87	42.6±4.38	44.8±4.45	1.73
		Kidney	23.6	22.5	24.6	27.9	
			31.4	30.4	36.2	35.3	
			19.9	19.2	21.9	21.2	
			19.7	18.5	22.1	21.9	
			19.9	17.9	22.5	23.3	
			26.6	25.7	26.6	26.8	
			28.7	26.6	29.5	29.8	
		Mean..	24.3±4.75	23.0±4.76	26.2±5.21	26.6±5.00	
		Supra-renal	302.1	304.2	312.5	272.2	
			345.8	370.8	341.7	421.1	
			395.8	387.5	375.0	366.7	
			352.0	324.0	356.0	337.6	
			304.4	330.4	308.7	265.0	
			286.0	280.0	300.0	298.4	
			332.5	340.0	337.5	325.0	
		Mean..	331.2±37.48	333.8±36.90	333.1±27.35	326.6±55.09	
		Pancreas	10.6	10.4	11.5	11.3	
			11.9	12.8	12.1	11.8	
			8.1	7.1	8.8	8.4	
			8.0	7.1	8.6	7.9	
			5.5	5.3	7.4	6.7	
		Mean...	8.8±2.49	8.5±3.01	9.7±2.02	9.2±2.22	
		Spleen	34.4	34.2	60.4	69.1	
			29.1	26.6	46.6	58.1	
			42.5	38.3	60.1	71.4	
			26.1	28.8	51.6	53.3	
		Mean..	33.0	32.0	54.7	63.0	
		Brain	57.6	51.8	55.4	52.4	
			54.5	55.2	57.2	57.7	
		Mean..	56.1	53.5	56.3	55.1	

ed 42.2 ± 4.87 mg.ASA/100 g. and 44.8 ± 4.45 mg.TASA/100 g. The figures for the kidney were 23.0 ± 4.76 mg.ASA/100 g. and 26.6 ± 5.00 mg.TASA/100 g.; for the brain they were 53.5 mg.ASA/100 g. and 55.1 mg.TASA/100 g. The ASA and TASA values in the spleen of the female rats were slightly higher than in the male rats, the figures being 32.0 mg.ASA/100 g. and 63.0 mg.TASA/100 g. The TASA concentrations of blood in both sexes were higher than normals, the value being about 1.7 mg/100 ml. In the pancreas the ascorbic acid appeared to have returned to the normal levels in both sexes. In the suprarenals the concentrations of ASA and TASA in the male rat were 365.4 ± 55.04 mg.ASA/100 g. and 375.5 ± 65.75 mg.TASA/100 g., the corresponding values in the female rat being 333.8 ± 36.90 mg.ASA/100 g. and 326.6 ± 55.09 mg.TASA/100 g. The ascorbic acid concentrations in the suprarenals seem to have increased when compared with the previous groups in Tables 8 and 9 but are still below the normal levels. It appears that most of the ascorbic acid introduced into the system has been disposed of during the 24 hours following the injection (vide Table 17).

The changes brought about in the animals injected with DHA on two successive days and sacrificed after an interval of 24 hours, are shown in Tables 12 and 13. Apart from slight variations in the individual organs, the general picture of the heightened concentration of ascorbic acid, as observed in the previous group of experiments, is still apparent. In the male rats the liver contained 50.0 mg.ASA/100 g. and 58.0 mg.TASA/100 g. These are slightly higher than those in the previous group. The kidneys contained/

Table 12.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the male rats following two injections of dehydroascorbic acid. Tissues removed after an interval of 24 hours from the last injection.

No.	Body Weight Initial g.	Body Weight Final g.	Tissue	Indophenol method with unbuffered extract mgASA/100g.	Indophenol method with buffered extract mgASA/100g.	Bolin & Book method mgTASA/100g.	Roe & Kuether method mgTASA/100g.	Whole blood (R & K method) mgTASA/100ml.	Blood sugar mg/100ml.
1	160	132	Liver	51.8	49.7	50.0	59.4	1.51	114
	160	130		49.7	50.2	54.1	56.6	2.35	140
			Mean ..	50.8	50.0	52.1	58.0		
			Kidney	31.6	29.7	33.0	30.0		
				24.9	23.6	30.4	29.2		
			Mean ..	27.8	26.7	31.7	29.6		
			Supra-renal	422.2	427.8	438.9	481.3		
				425.0	429.2	458.3	434.1		
			Mean ..	423.6	428.5	448.6	457.7		
			Pancreas	15.7	15.2	17.5	16.8		
				12.4	11.4	12.4	11.9		
			Mean..	14.1	13.3	15.0	14.4		
			Spleen	57.8	58.3	75.0	-		
				68.5	72.7	85.9	-		
			Mean ..	63.7	65.5	80.5			

Table 13.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the female rats following two injections of dehydroascorbic acid. Tissues removed after an interval of 24 hours from the last injection.

No.	Animal Weight Initial g.	Final g.	Tissue	Indophenol method without buffered extract mgASA/100g.	Indophenol method with buffered extract mgASA/100g.	Bolin & Book method mgTASA/100g.	Roe & Kuether mgTASA/100g.	Whole blood mgTASA/100ml.	Blood sugar mg/100ml.
1	155	135	Liver	42.1	40.5	39.2	35.9	1.41	131
2	160	132		41.2	39.7	38.6	41.9	1.47	233
			Mean .	41.7 . . .	40.1 . . .	38.9 . . .	38.9		
			Kidney	29.3	23.2	23.7	25.4		
				21.7	19.5	20.0	19.3		
			Mean	25.5 . . .	21.4 . . .	21.9 . . .	22.4		
			Supra-renal	455.2	448.3	465.5	430.8		
				307.1	271.4	360.7	357.4		
			Mean	381.2 . . .	359.9 . . .	413.1 . . .	394.1		
			Pancreas	11.2	8.7	9.5	8.4		
				10.9	9.4	10.1	9.9		
			Mean	11.1 . . .	9.1 . . .	9.8 . . .	9.2		
			Spleen	86.8	90.3	79.9			
				66.9	60.7	77.0	69.8		
			Mean	76.9 . . .	75.5 . . .	78.5 . . .	69.8		

contained 26.7 mg.ASA and 29.6 mg.TASA per 100 g., in the pancreas the values were 13.3 mg.ASA and 14.4 mg.TASA per 100 g. The spleen in these animals showed a marked increase in ascorbic acid concentrations, the values being 65.5 mg.ASA and 80.5 mg.TASA per 100 g. (by the Bolin and Book method). However, in the female rats, a tendency towards a decrease in concentrations in the liver and kidney is noticeable. The liver of the female rats contained 40.1 mg.ASA/100 g. and 38.9 mg.TASA/100 g., the values in the kidney being 21.4 mg.ASA and 22.4 mg.TASA per 100 g. The concentrations in the suprarenals have increased slightly beyond the normal levels. The suprarenal in the male rat contained 428.5 mg.ASA and 457.7 mg.TASA per 100 g., the corresponding figures for the female being 359.9 mg.ASA and 394.1 mg.TASA per 100 g. The values in the pancreas were 9.1 mg.ASA and 9.2 mg.TASA per 100 g., while in the spleen they were 75.5 mg.ASA and 78.5 mg.TASA per 100 g. (Bolin and Book method). The blood TASA was still maintained at a high level. The blood sugar was estimated in these animals at the time of sacrifice. Of the four animals, one was definitely hyperglycaemic with a blood sugar of 233 mg/100 ml. while two others had slightly high sugar levels in blood (vide Table 17). A remarkable feature of these results is the fact that there has not been a marked change in the concentrations of ascorbic acid in the tissues following the second dose. Possibly in the meantime, the mechanism of disposal has become too efficient to deal with the extra amount of ascorbic acid that was introduced.

Table/

Table 14 summarises the results obtained in acute experiments in female rats following the third injection of DHA. In these experiments, the ASA concentrations were estimated by the indophenol method using unbuffered extracts while the TASA values were obtained by the Roe and Kuether method. The suprarenal glands, however, were pooled and analysed. Some of the TASA values in these estimations, in the liver especially, are lower than the ASA values, possibly due to interference in the methods. Following the injection it will be noted that a rise in the ascorbic acid (ASA + TASA) concentrations has taken place (Cf. Table 9). Of the TASA concentrations of 278.8 mg./100 g. in the liver and 657.1 mg./100 g. in the kidney, there was complete reduction in the liver, whereas in the kidney the reduction was about 50 per cent as before, the value being 328.3 mg.ASA/100 g. In the voluntary muscle of the TASA concentration of 27.8 mg/100 g., the ASA was 15.5 mg./100 g. representing about 50 per cent reduction. The concentrations in the suprarenals of ASA were 396.5 mg/100 g. and TASA, 363.1 mg/100 g. These figures are not significantly higher than normal. The blood sugar was estimated immediately before the injection of DHA in these animals. Hyperglycaemia was present in all of them except two. Mention must be made of the fact that the tissues have retained the power of reducing, and doing that with extreme rapidity, even though they had been subjected to so much strain in the meanwhile.

The results of the chronic experiments following the three injections of DHA are summarised in Tables 15 and 16. In these experiments, the animals/

Table 14

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the female rats following three injections of dehydroascorbic acid. Tissues removed 15 minutes after the completion of the third injection.

No.	Weight in g.	Tissue	Indophenol method without buffered extract mgASA/100g.	Roe & Kuether method mgTASA/100g.	Blood Sugar mg/100 ml.	
1	145	Liver	317.5	234.4	402	
3	130		277.1	285.5	125	
4	140		210.4	593.1	338 *	
8	145		250.0	269.1	214	
7	132		262.7	308.2	363	
9	135		323.7	301.9	328	
11	145		306.0	273.7	145	
			Mean 289.5	278.8		
				Reduction .. complete		
3		Kidney	322.6	583.3		
8			277.9	600.8		
7			347.8	781.3		
9			355.0	662.2		
10	140		338.1	657.1	230	
			Mean 328.3	657.0		
				Reduction ... 50%		
		Muscle	15.5	27.8		
				Reduction ... 55%		
		Adrenals	383.0	389.4		Mixed rats 8,7
			410.0	336.8		" " 9,
						10 & 11.
			Mean 396.5	363.1		

* Animal died in 3 mins. after last injection. (Excluded from mean)

Table 15.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the male rat following three injections of dehydroascorbic acid. Tissues removed after an interval of 8 days or more.

No.	Body Weight Initial g.	Body Weight Final g.	Tissue	Indophenol method without buffered extract mgASA/100g.	Indophenol method with buffered extract mgASA/100g.	Bolin & Book method mgTASA/ 100g.	Roe & Kuether method mgTASA/ 100g.	Whole blood R & K method mgTASA/ 100 ml.	Blood Sugar mg/100 ml.	Day of Sacrifice
1	165	185	Liver	46.3	45.1	43.9	-	-	135	8th
2	176	173		38.8	39.4	37.5	36.3	0.62	119	8th
3	175	180		39.5	41.1	35.1	39.8	0.7	119	9th
4	175	195		50.7	45.2	43.3	40.4	0.7	162	10th
5	179	194		42.8	41.7	39.5	36.2	0.68	122	11th
6	200	205		35.2	34.2	34.5	38.9	0.73	155	15th
			Kidney	29.5	28.5	27.7	-			
				16.8	16.4	17.8	17.0			
				20.8	20.1	19.7	20.1			
				22.4	20.6	23.3	23.2			
				22.9	21.9	21.6	19.7			
				16.6	16.3	18.8	17.6			
1			Supra-	320.0	320.0	310.0	-			
2			renal	400.0	435.0	405.0	411.1			
3				384.2	400.0	342.1	359.4			
4				375.0	407.5	370.0	396.1			
5				485.0	480.0	450.0	420.6			
6				423.8	439.1	395.2	367.5			
			Pancreas	15.2	15.0	14.7	-			
				8.9	9.7	10.1	9.8			
				9.9	9.1	9.0	8.8			
				11.6	10.5	10.8	10.5			
				10.9	9.9	10.2	9.3			
				9.1	9.2	9.7	9.4			
1			Spleen	31.0	29.2	39.8	-			
2				25.2	28.5	35.1	41.0			
				43.9	45.8	49.0	49.2			
				45.5	38.6	45.5	41.5			
				29.0	27.5	34.4	36.9			
				30.0	28.0	39.3	45.9			

Table 16

Concentrations of ascorbic acid and total ascorbic acid in the tissues of the female rats following three injections of dehydroascorbic acid. Tissues removed after an interval of 8 days or more.

No.	Animal Weight Initial g.	Final g.	Tissue	Indophenol method without buffered extract mgASA/100g.	Indophenol method with buffered extract mgASA/100g.	Bolin & Book method mgTASA/100g.	Roe & Kuether method mgTASA/100g.	Whole Blood Roe & Kuether mgTASA/100ml.	Blood sugar mg/100ml.	Day of sacrifice
1	160	160	Liver	24.7	22.1	24.7	29.9	0.44	96	8 days
2	150	138		25.2	22.2	24.6	23.6	0.45	333	8 days
3	150	137		17.9	15.5	17.4	18.2		399	10 days
			Kidney	15.7	12.8	15.5	14.4			
				12.3	8.4	11.5	10.1			
				12.3	8.8	12.1	10.8			
			Supra-renals	400.0	400.0	430.0	425.0			
				268.8	264.1	318.8	323.9			
				440.7	437.0	463.0	456.5			
			Pancreas	9.4	8.2	8.5	9.1			
				10.7	8.0	10.7	11.8			
				9.4	9.6	10.5	9.5			
			Spleen	21.6	20.4	31.8	42.1			
				21.3	19.5	28.8	37.8			
				21.0	21.0	28.4	31.8			

animals were sacrificed after an interval of 8 days or more following the last injection. It will be observed that the concentrations of ascorbic acid in the tissues and blood have fallen and reached almost the original levels. Thus in the male rat, sacrificed on the 8th day, the liver was found to contain 36.3 mg.TASA/100 g. (Roe and Kuether method) and the corresponding figures for the kidney, suprarenal, pancreas and spleen in the same animal were 17.0 mg., 411.1 mg., 9.8 mg. and 41.0 mg.TASA per 100 g. respectively. Similarly in a female rat, the corresponding figures, for liver, kidney, suprarenals, pancreas and spleen, were 29.9 mg., 14.4 mg., 425.0 mg., 9.1 mg. and 42.1 mg.TASA per 100 g. respectively. The blood TASA values in the above animals were 0.62 mg. and 0.44 mg. per 100 ml. The blood sugar was estimated at the time these animals were sacrificed. Of the nine animals, two were hyperglycaemic with high sugar values while three others had slightly high sugar levels. From the results, it appears that the organism has recovered or is recovering from the strains to which the metabolism of ascorbic acid, in general, was subjected.

In some of the experiments, already described (Tables 10 to 13, 15 and 16) following one, two and three injections of DHA, the animals were sacrificed after an interval of 24 hours or more. The changes in the concentrations of ascorbic acid when compared with the normal control are summarised in Table 17 for easy reference. Only the TASA values obtained in the organs have been taken into account for the preparation of this table./

Table 17.

Changes in tissue concentrations of ascorbic acid observed after an interval following DHA injections.

Male Rats	Liver	Kidney	Supra-renal	Pancreas	Spleen	Brain	Whole blood
	mgTASA per 100g. of tissue or 100 ml. of blood (Roe & Kuether method).						
After 24 hrs following one injection of DHA	47.6	30.1	375.5	12.5	49.9	61.2	1.705
Control	31.9	14.4	393.8	12.7	41.9		
Difference	15.7	15.7	-	-	8.0		
After 24 hrs. following 2 injections of DHA	58.0	29.6	457.7	14.4	80.5 (B&B)		
Control	31.9	14.4	393.8	12.7	41.9		
Difference	26.1	15.2	+	1.7	38.6		
After 8 days following 3 injections of DHA	36.3	17.0	411.1	9.8	41.0		
Control	31.9	14.4	393.8	12.7	41.9		
Difference	4.7	2.6	+	-	-		
Female Rats							
After 24 hrs following one injection of DHA	44.8	26.6	326.6	9.2	63.0	55.1	1.73
Control	23.9	10.6	365.5	-	32.4	45.0	
Difference	20.9	16.0	-		30.6	10.1	
After 24 hrs following 2 injections of DHA.	38.9	22.4	394.1	9.2	69.8		
Control	23.9	10.6	365.5		32.4		
Difference	15.0	11.8	+		37.4		
After 8 days following 3 injections of DHA	29.9	14.4	425.0	9.1	42.1		
Control	23.9	10.6	365.5		32.4		
Difference	6.0	3.8	+		9.7		

table. The differences between the mean values denote the changes brought about in the concentrations.

In view of the observations made in the experiments with DHA, it was of interest to know the immediate effects of ascorbic acid under similar conditions. Ascorbic acid was dissolved in 0.9 per cent sodium chloride solution in the proportions used for the preparation of DHA and was injected intravenously to rats starved for 24 hours. As in the experiments with DHA, a preliminary dose of 0.2 g/Kg. followed by another dose of 0.7 g/Kg. body weight of ascorbic acid was administered. Previous to the larger dose of ascorbic acid, nembutal in a dose of 6 mg/100 g. was injected intraperitoneally. Following the larger dose of ascorbic acid, the animals were bled from the 10th to the 14th minute and tissues were removed from the 15th to the 21st minute. A male and a female rat were used for these experiments and the results obtained are summarised together in Table 18. It will be observed that the values obtained by the indophenol method in the cases of liver, suprarenals, pancreas and spleen agree well with those obtained by the two phenylhydrazine methods. But in the case of the kidneys the ASA values are much higher than the TASA values obtained by the Roe and Kuether method. For the description of results, the TASA values obtained by the Roe and Kuether method are being considered. It will be noted that the greatest concentration of ascorbic acid has taken place in the kidneys. Thus, in the male rat the kidney was found to contain 624.6 mg.TASA/100 g. This represented a rise of about 40 times/

Table 18.

Concentrations of ascorbic acid and total ascorbic acid in the tissues of a male and a female rat injected with ascorbic acid (0.2g. and 0.7g. per Kg. body wt). Tissues removed 15 minutes after the completion of the injection.

Sex	Weight	Tissue	Indophenol method without buffered extract mgASA/ 100g.	Indophenol method with buffered extract mgASA/ 100g.	Bolin & Book method mgTASA/ 100g.	Roe & Kuether method mgTASA/ 100g.	Whole blood Roe & Kuether method mg/100 ml.
Male	172g.	Liver	128.3	122.7	122.7	130.7	139.0
Female	150g.		153.0	156.3	146.4	168.1	162.0
		Kidney	699.5	739.5	720.5	624.6	
			731.5	722.7	696.6	651.6	
		Supra-renal	273.8	273.8	314.3	326.5	
			350.0	333.3	374.1	352.1	
		Pancreas	110.9	107.3	119.4	126.1	
			153.4	147.7	150.0	142.5	
		Spleen	78.3	71.0	91.0	84.2	
			73.1	65.9	88.8	91.0	

times the original level. The kidney in the female rat contained 651.6 mg.TASA/100 g., a value which was about 60 times the normal level. In the liver, the male rat was found to contain 130.7 mg.TASA/100 g. representing a fourfold rise and in the female a sevenfold increase had resulted in a concentration of 168.1 mg.TASA/100 g. The pancreas in the male rat had 126.1 mg.TASA/100 g.; in the female it contained 142.5 mg.TASA/100 g. These represented a 10 to 16 fold rise from the original levels. The concentrations in spleen had gone up two to threefold to reach the levels of 84.2 mg.TASA/100 g. in the male and 91.0 mg.TASA/100 g. in the female rat. The TASA levels in blood were 139.0 mg. and 162.0 mg. per 100 ml. in the male and female respectively. The suprarenal in the male rat was found to contain 326.5 mg.TASA/100 g., the corresponding figure in the female was 352.5 mg.TASA/100 g. When these figures are compared with the normals they appear to be low. When the results obtained in the acute experiments with DHA in Tables 8 and 9 are compared with those above, the following features are noticeable. Following the ascorbic acid injection, a greater concentration of TASA has taken place in the blood. The concentrations in the pancreas are a little higher; they are approximately equal in both cases in the kidney. The rise in concentrations in the liver and spleen is less marked. The fall in the levels of ascorbic acid in the suprarenals is less marked, but, since in other tissues an actual increase is found, this is significantly outstanding behaviour; and it is presumably related to the peculiarities of adrenal cortex metabolism in response to emotional and/

and other stimuli.

It may be important that ASA is concentrated in the pancreas much more efficiently than is DHA, while DHA is more avidly taken up in the liver, spleen and other tissues.

DISCUSSION

The methods of estimation of ascorbic acid and total ascorbic acid used in this investigation, namely the indophenol and the phenylhydrazine methods, are well known analytical procedures. From the results it appears that, apart from experimental errors, the question of interference by some metabolite or metabolites in one or the other or in both the methods cannot be ruled out. Of the tissues examined, the interference has been observed most frequently in the liver, kidney, suprarenals and brain but not in all at the same time. Each of these organs is of high metabolic activity.

The differences in the tissue concentrations of ascorbic acid observed in the sexes of the control animals are most probably due to unequal rates of growth in the male and female rat. In the range of body weight of the rats used in this study, the male rat generally gained weight more rapidly. Thus days and sometimes weeks passed before the female rats attained the weight of the corresponding male rat. It may be recalled that in the control animals the body weights were almost equal in the two sexes and as such the female rats were relatively more mature than the male rats. Bessey and King (77) observed that under identical conditions of nutrition, the tissues of the young and rapidly growing animals tend to have a higher vitamin C concentration. A few other observations in this connection deserve mention. Todhunter and McMillan (85) demonstrated a significantly higher concentration of ascorbic acid in plasma and blood of the adult male rat. Salhanick, Zarrow/

Zarrow and Zarrow (86) found an increase in ascorbic acid concentration of the pituitary with increase in age in the female rat. Sutton, Kaeser and Hansard (87) observed a sex difference in the rate of excretion of vitamin C. The female rats excreted only about half as much as did the male rats. A close relationship exists between growth and the metabolism of ascorbic acid; the disappearance of the vitamin is greater during periods of rapid growth. Reid (88) observed in the guinea pig, that the younger the animal and the more rapidly it grows, the smaller is the amount of vitamin C excreted in the urine.

Following nembutal anaesthesia, in the rat, a depletion of adrenal ascorbic acid takes place and simultaneously the ascorbic acid concentrations in the liver and kidney rise, particularly in the male rat. This observation is in line with a similar observation made by Bowman and Muntwyler (79) in the albino rats following ether anaesthesia. They reported that along with this change in the concentrations, there is increased excretion of ascorbic acid in the urine of a short duration. Ludewig and Chanutin (89) observed a delayed depletion of adrenal ascorbic acid following nembutal anaesthesia. Recent observations, in man, have shown that the induction of general anaesthesia with pentothal, cyclopropane and ether was associated with increased levels of 17-hydroxycorticosteroids in blood plasma (90). It appears that this rise in concentration of 17-hydroxycorticosteroids in plasma is due both to the increased secretion by the adrenal cortex and to the impaired destruction in the liver temporarily (91). It is also known that conjugated products/

products of 17-hydroxycorticosteroids are excreted in the urine in large quantities under the above conditions. The relation of ascorbic acid to this synthesis and destruction of corticosteroids is at present obscure.

Dehydroascorbic acid tends to diffuse readily into the liver, spleen, pancreas, kidney, brain and muscle in the rat. It is reduced in the liver to the extent of about 80 per cent within a short time. Probably liver is the site where most of the added dehydroascorbic acid is reduced and the study of the literature suggests the same. The amount of reduction in the spleen is a little less, whereas in the pancreas, kidney and muscle about half as much is reduced during the same period. Patterson et al.(51) observed that, with gradual accumulation of reduced ascorbic acid, the rise in the level in the brain tends to fall off. The dosage of dehydroascorbic acid used in this investigation was very high, and it is conceivable that the tissues were more or less saturated with ascorbic acid from the reduction of dehydroascorbic acid. The excess was circulating in the blood and was being concentrated in the kidneys. The very high concentration in the kidneys is probably an expression of this situation. It is also an attempt on the part of the organism to throw out the excess in the urine before it could be converted to other metabolic products such as CO_2 and others. During the first 24 hours following the injection, most of the ascorbic acid introduced disappears and only a little remains. Following the second dose, presumably there occurred a rise in the levels and the process of reduction was repeated. At the end of 24 hours following this, a rise in the concentration/

trations in the tissues above the levels obtained after the first dose was expected. Such a rise was not observed. Possibly in the meantime the metabolic rate as a whole has become geared to a high level and the organism is too efficient in the disposal of the extra amount. When time is allowed, the increased levels tend to return to their original values.

The mechanism of reduction almost certainly involves glutathione and the cycle goes into action every time the dose is repeated. Borsook et al. (19) from their studies concluded that glutathione is possibly the main agent concerned in the reduction of dehydroascorbic acid in vitro and in vivo. Schultze et al. (41) suggested that glutathione and fixed -SH compounds are the main agents responsible for the reduction of added dehydroascorbic acid. It is interesting to note that their observation of a large excess of ascorbic acid slowing down the rate of reduction of DHA in vitro bears a similarity to the observation of Patterson et al. (51) mentioned above. Bhattacharya (59) has demonstrated a fall in the glutathione concentration in the rat liver along with reduction of dehydroascorbic acid. A significant change in the concentrations of glutathione in the pancreas was not observed in that investigation. Suggestive evidences of reduction with consequent damage to the islet-cells have been put forward by Patterson and Lazarow (64) and Bhattacharya (59).

From the data collected in these experiments it is not possible to draw a complete picture of the fate of the injected dehydroascorbic acid. From the observations it appears that major amounts of the injected dose were/

were reduced to ascorbic acid. Dehydroascorbic acid is not stable at the temperature and pH of the tissues for more than a few minutes and undergoes irreversible transformation to diketogulonic acid. Penney and Zilva (20) reported that, of the dehydroascorbic acid injected intramuscularly into the guinea pig, about 25 per cent was excreted as diketogulonic acid during the next 24 hours, mostly during the first 3 or 4 hours. Some of the injected dehydroascorbic acid in these rats must have been excreted in this way. Burns et al. (25), from their tracer studies with labelled ascorbic acid, concluded that, of the amount synthesized in the normal rat, only about 15 per cent was excreted in the urine during the 24 hours. In a rat, stimulated to synthesize ascorbic acid with chloretone, as much as 43 per cent could be excreted. They found that significant amounts were converted to CO_2 and expired. In the urine appreciable quantities of oxalate were present besides ascorbic acid, dehydroascorbic acid, diketogulonic acid and other unidentified products. In the present study possibly the metabolic processes were enhanced, and large quantities of ascorbic acid were disposed of in a manner analogous to the above. In some of the animals dysenteric symptoms were noted. It might have been a result of increased excretion through the gastrointestinal tract. Reid (92) reported that following intraperitoneal injection of ascorbic acid into guinea pigs the amount of ascorbic acid excreted into the stomach and intestine was much more than 15 per cent of the injected dose.

The causation of diabetes appears to be incidental in the situation brought/

brought about by the exhibition of large amounts of dehydroascorbic acid. It is doubtful whether a similar situation could occur naturally. Once the organism has dealt with the situation, the metabolism of ascorbic acid as a whole returns to normal. In the context of the picture drawn above, the observation of diketogulonic acid in blood in human diabetes (93) does not fit in.

The role of the suprarenal gland in this requires further study. The observations made, suggest that intravenous injections of dehydroascorbic acid or ascorbic acid, at the dosages used in this investigation, induce a stressful condition and immediate depletion of ascorbic acid from the adrenal gland ensues. The real mechanism of this depletion is not understood at present. It is said that, most probably, ascorbic acid undergoes destruction within the gland. It is reasonable to assume that the concentration of ascorbic acid inside the gland falls. Even with this reduced concentration inside the gland, the dehydroascorbic acid or ascorbic acid, injected and present in high concentration in the blood outside, does not seem to diffuse into the gland, at least at a sufficient rate to replace completely the amount metabolised. The suggestion of Patterson and Mastin (51) that ascorbic acid must be oxidised to dehydroascorbic acid before it penetrates the cell membrane does not seem to hold good in this instance. The subsequent events in the suprarenals follow the pattern of events in stress. The adrenal ascorbic acid tends to rise and the weight of the gland increases (vide appendix). Dury (94) observed a significant rise in adrenal ascorbic acid 65 hours after alloxan injection/

injection together with an increase in the weight of the adrenal glands in the albino rat. These observations are strongly suggestive of the fact that the phenomenon of stress plays a great role at the initial stages following the injections of these diabetogenic substances. Once we accept that this is so, the chain of reactions set in motion becomes too complex to visualise. Dehydroascorbic acid and alloxan both show the triphasic response in the blood sugar, hyperglycaemia, hypoglycaemia and hyperglycaemia. The sequence of events, in terms of stress, would appear to be as follows

Injection of DHA or Alloxan → Stress + Damage to the beta-cells

Stress → hyperglycaemia due to adrenocortical causes (? Role of Pituitary)

→ hypoglycaemia

→ hyperglycaemia, possibly due to the failure of the pancreas to elaborate insulin.

Transient hyperglycaemia following DHA injection is possibly mainly due to adrenocortical/

adrenocortical causes. Carey, Vollmer, Zwemer and Spence (95) observed a fall in the adrenal ascorbic acid and cholesterol concentrations, following subcutaneous or intraperitoneal injections of glutathione in large doses in rats and guinea pigs. It has been observed that following intravenous injection of ascorbic acid, there is a fall in the adrenal ascorbic acid concentration. It has been claimed that ascorbic acid potentiates the diabetogenic effect of alloxan. Levey and Suter (96) expressly stated that for this potentiation to occur, the intravenous injection of ascorbic acid should be followed within a minute by alloxan. Patterson (61) observed a synergistic action of dehydroascorbic acid or ascorbic acid with alloxan and the conditions, under which he observed this synergism, were almost similar. The administration of pituitary adrenocorticotrophic hormone (ACTH) is followed by a temporary decrease in the amount of ascorbic acid present in the adrenal glands (81, 97). In the light of these evidences, the interpretation of the observations made at the initial stages of the reaction becomes very difficult. Some active agent or agents ("neurohumour", hypothalamus) appear to be involved in common with all these substances, at least at the initial stages of the reaction.

SUMMARY AND CONCLUSIONS

1. Observations were made on the ascorbic acid and "total ascorbic acid" (ASA + DHA + DKA) concentrations in the liver, kidney, pancreas, spleen, suprarenals and other tissues before and after the intravenous injection of dehydroascorbic acid in diabetogenic doses to the male and female albino rats. The immediate effects following the intravenous injection of ascorbic acid under similar conditions were also studied.

2. Immediately following the dose of dehydroascorbic acid the concentration of total ascorbic acid rose most in the kidneys, where about a fiftyfold rise was noted. The concentrations rose in other tissues in the following way, liver (10-16 fold), pancreas (7-10 fold) and spleen (about fourfold). The dehydroascorbic acid that entered the tissues was rapidly reduced. The extent of reduction in the liver was more than 80 per cent, it was a little less in the spleen whereas in the kidneys and pancreas about 50 per cent reduction took place. Twenty four hours later most of the excess ascorbic acid had disappeared, though slightly high levels were observed in some of the tissues, specially in the liver and kidneys. Following the second dose, the concentrations in the tissues presumably rose but at the end of twenty four hours further accumulation had not taken place. Following the third dose reduction to ascorbic acid was observed to take place. The concentrations in the tissues were almost normal eight days later.

3./

3. Compared to dehydroascorbic acid, the administration of ascorbic acid under similar conditions led to a greater rise in the concentrations in the pancreas; the kidneys concentrated to the same extent as before whereas the liver and spleen concentrated much less.

4. After preliminary desensitisation, the intravenous injection of 0.7 g/Kg of dehydroascorbic acid caused an immediate depletion of adrenal ascorbic acid. The administration of ascorbic acid under similar conditions resulted in depletion of adrenal ascorbic acid as well. The fall was more marked after dehydroascorbic acid and in the male rat. Twenty four hours later a restoration in concentration of adrenal ascorbic acid occured together with a significant rise in adrenal weight.

5. The relationship between the causation of experimental diabetes and ascorbic acid metabolism in general is discussed. After the onset of experimental diabetes the ascorbic acid metabolism is not necessarily deranged. The role of the suprarenal glands and the phenomenon of stress at the initial stages of the production of experimental diabetes requires elucidation.

APPENDIX

The weights of the adrenal glands obtained from the animals used in the different groups of experiments described in the text are summarised in Tables 19 and 20. In Groups A in the tables are placed, the control animals which were sacrificed by stunning. The mean ±S.D. of the adrenal (mg/100 g. body weight) in the male animals were 19.5 ± 2.03 mg., the corresponding figure in the females was 26.6 ± 5.12 mg. The Groups B show the control animals sacrificed under nembutal anaesthesia. The corresponding figures, as above, were 18.1 ± 1.04 mg. in the male and 28.6 ± 2.40 mg. in the female. In Groups C, D and E are the animals which were starved for 24 hours preparatory to the injection experiments. Groups C show the animals used in the acute experiments with DHA and ASA. The mean ±S.D. of the adrenal weights (mg/100 g. body weight after starvation) were, in the male 21.3 ± 1.52 mg. and 32.3 ± 2.87 mg. in the female. In Groups D are summarised the adrenal weights of the animals sacrificed after an interval of 24 hours, following the injection of DHA. The means ±S.D. of the adrenal weight (mg/100 g. final body weight) were 24.2 ± 1.92 mg. and 36.7 ± 2.90 mg. in the male and female rats respectively. Statistical analyses of the data in Groups C and D show that the weights of the adrenal glands in Group D are significantly higher than the weights in Group C (P lies between 0.05 and 0.02 in both sexes). The animals sacrificed after 24 hours following two injections of DHA, as shown in Groups E, had the mean adrenal weights of 30.6 mg. in the male and 48.7 mg. in/

in the female rats respectively. The values are higher than those corresponding in Groups D. It is much regretted that the significance of the findings were realised late in the investigation and much material had been lost in the meantime.

Table 19.

Variations in the weights of adrenal glands in the male rat.

Groups	Body weight in g. Initial	Final	Adrenal weight in mg.	Adrenal weight 100g. initial wt.	Adrenal weight 100g. final wt.
A.	165		32	19.4	
	163		28	17.2	
Control animals	165		31	18.8	
sacrificed by	170		37	22.9	
stunning	160		33	20.6	
	155		28	18.1	
			Mean	19.5 ± 2.03	
B.	160		27	16.9	
	150		30	20.0	
Control animals	160		29	18.1	
sacrificed under	170		31	18.2	
nembutal anaesth-	155		28	18.1	
esia	160		28	17.5	
			Mean	18.1 ± 1.04	
C.	160	145	28	17.5	19.3
Animals used for	165	160	34	20.6	21.3
acute experiments	160	140	30	18.8	21.4
with DHA and ASA	172	165	38	22.1	23.0
			Mean	19.75 ± 2.02 . .	21.3 ± 1.52
	168		29	17.3	
D.	150	140	33	22.0	23.6
	150	146	39	26.0	26.7
Animals injected	150	142	33	22.0	23.2
with DHA once and	150	144	37	24.7	25.7
sacrificed 24 hrs.	160	150	33	20.6	22.0
later.			Mean	22.1 ± 3.08 . .	24.2 ± 1.92
E.	160	132	34	21.3	25.8
Animals injected	160	130	46	28.8	35.4
twice with DHA and			Mean	25.8 . .	30.6
sacrificed 24 hrs. later.					

Table 20.

Variations in the weight of adrenal glands in the female rat.

Groups	Body weight in g. Initial	Final	Adrenal weight in mg.	Adrenal weight 100g. initial wt.	Adrenal weight 100g. final wt.
A.	150		39	26.0	
	140		26	18.6	
Control animals	178		48	30.0	
sacrificed by	175		42	24.0	
stunning	160		44	27.5	
	155		52	33.5	
			Mean . . .	26.6 ± 5.12	
B.	160		48	30.0	
	170		56	32.9	
Control animals	160		44	27.5	
sacrificed under	160		43	26.9	
nembutal anaesth-	158		44	27.8	
esia.	165		44	26.7	
			Mean . . .	28.6 ± 2.40	
C.	160	150	47	29.4	31.3
	160	148	50	31.3	33.8
Animals used for	163	152	44	27.0	28.9
acute experiments	150	141	44	29.3	31.2
with DHA and ASA	155	140	51	32.9	36.4
			Mean . . .	30.0 ± 2.24 . .	32.3 ± 2.87
D.	150	135	51	34.0	37.8
	157	140	43	27.4	30.7
Animals injected	155	145	54	34.8	37.2
with DHA once	160	148	58	36.3	39.2
and sacrificed	160	146	53	33.1	36.3
24 hours later.	162	143	56	34.6	39.2
	155	138	50	32.3	36.2
			Mean . . .	33.2 ± 2.86 . .	36.7 ± 2.90
E.	155	135	68	43.9	50.4
Animals injected	160	132	62	38.8	47.0
twice with DHA			Mean . . .	41.4 . .	48.7
and sacrificed					
24 hours later.					

BIBLIOGRAPHY

1. Szent-Györgyi, A., Biochem. J. 22, 1387, (1928).

2. Tillmans, J. and Hirsch, P., Biochem. Z. 250, 312, (1932). Cited by Lloyd, B.B. and Sinclair, H.M. in Biochemistry and Physiology of Nutrition. Edited by Bourne, G.H. and Kidder, G.W. Vol. 1. p.371, (1953). Academic Press Inc., New York.

3. King, C.G. and Waugh, W.A., Science. 75, 357, (1932).
King, C.G. and Waugh, W.A., J. Biol. Chem. 97, 325, (1933).

4. Svirbely, J.L. and Szent-Gyorgyi, A., Nature. 129, 576, (1932).
Svirbely, J.L. and Szent-Györgyi, A., Biochem. J. 27, 279, (1933).

5. Szent-Gyorgyi, A. and Haworth, W.N., Nature, 131, 24, (1933).

6. Harris, L.J. and Ray, S.N., Biochem. J. 27, 580, (1933).

7. Hirst, E.L. and Zilva, S.S., Biochem. J. 27, 1271, (1933).

8. Reichstein, T., Grüssner, A. and Oppenauer, R., Helv. Chim. Acta. 16, 1019, (1933). Cited by Lloyd, B.B. and Sinclair, H.M in Biochemistry and Physiology of Nutrition. Edited by Bourne, G.H. and Kidder, G.W. Vol. 1. p.372, (1953). Academic Press Inc., New York.

9. Ault, R.G., Baird, D.K., Carrington, H.C., Haworth, W.N., Herbert, R.W., Hirst, E.L., Percival, E.G.V., Smith, F. and Stacey, M., J. Chem. Soc. (1933), 1419.

10. Herbert, R.W., Hirst, E.L., Percival, E.G.V., Reynolds, R.J.W., and Smith, F., J. Chem. Soc. (1933). 1270.

11. Johnson, S.W. and Zilva, S.S., Biochem. J. 27, 1280, (1933).

12. Horowitz, W.H., Doerschuk, A.P. and King, C.G., J. Biol. Chem. 199, 193, (1952).

13. Horowitz, W.H. and King, C.G., J. Biol. Chem., 200, 125, (1953).

14. Horowitz, W.H. and King, C.G., J. Biol. Chem., 205, 815, (1953).

15. Isherwood, F.A., Chen, Y.T. and Mapson, L.W., Biochem. J. 56, 1, (1954).

16./

16. Reid, M.E. in The Vitamins, Chemistry, Physiology, Pathology. Edited by Sebrell, W.H., Jr., and Harris, R.S. Vol. 1. p.302, (1954) Academic Press Inc., New York.

17. Szent-Györgyi, A., J. Biol. Chem. 90, 385, (1931).

18. Keilin, D. and Hartree, E.F., Proc. Roy. Soc. (London) B125, 171, (1938).

19. Borsook, H., Davenport, H.W., Jeffreys, C.E.P. and Warner, R.C., J. Biol. Chem. 117, 237, (1937).

20. Penney, J.R. and Zilva, S.S., Biochem. J. 37, 403, (1943).

21. Penney, J.R. and Zilva, S.S., Biochem. J. 39, 1, (1945).

22. Rosenfeld, B., J. Biol. Chem. 150, 281, (1943).

23. Damron, C.M., Monier, M.M. and Roe, J.H., J. Biol. Chem. 195, 599, (1952).

24. Burns, J.J., Burch, H.B. and King, C.G., J. Biol. Chem. 191, 501, (1951).

25. Burns, J.J., Mosbach, E.H. and Schulenberg, S., J. Biol. Chem. 207, 679, (1954).

26. Monier, M.M. and Weiss, R.J., Proc. Soc. Exptl. Biol. Med. 80, 446, (1952).

27. Monier, M.M. and Weiss, R.J., Proc. Soc. Exptl. Biol. Med. 81, 598, (1952).

28. Monier, M.M. and Weiss, R.J., Proc. Soc. Exptl. Biol. Med. 84, 93, (1953).

29. Lamden, M.P. and Chrystowski, G.A., Proc. Soc. Exptl. Biol. Med. 85, 190, (1954).

30. Clayton, B.E., McSwinney, R.R. and Prunty, F.T.G., Biochem. J. 58, 542, (1954).

31. Fox, F.W. and Levy, L.F., Biochem. J. 30, 208, (1936).

32. Lyman, C.M., Schultze, M.O. and King, C.G., J. Biol. Chem. 118, 757, (1937).

33. Mapson, L.W., Biochem. J. 39, 228, (1945).

34. Weissberger, A. and LuValle, J.E., J. Amer. Chem. Soc. 66, 700, (1944).

35. Barron, E.S.G., deMeio, R.R. and Klemperer, F., J. Biol. Chem. 112, 625, (1936).

36./

36. Stotz, E., Harrer, C.J., Schultze, M.O. and King, C.G., J. Biol. Chem. 122, 407, (1937-38).

37. Giri, K.V., Nature, 166, 441, (1950).

38. Kinsey, V.E., Amer. J. Ophthalmol. 33, 257, (1950).

39. Stewart, C.P., Horn, D.B. and Robson, J.S., Biochem. J. 53, 254, (1953).

40. Hopkins, F.G. and Morgan, E.J., Biochem. J. 30, 1446, (1936).

41. Schultze, M.O., Stotz, E. and King, C.G., J. Biol. Chem. 122, 395, (1937-38).

42. Mapson, L.W. and Goddard, D.R., Nature. 167, 975, (1951).

43. Conn, E.E. and Vennesland, B., Nature. 167, 976, (1951).

44. Rall, J.W. and Lehninger, A.L., J. Biol. Chem. 194, 119, (1952).

45. Johnson, S.W. and Zilva, S.S., Biochem. J. 28, 1393, (1934).

46. Fox, F.W. and Levy, L.F., Biochem. J. 30, 211, (1936).

47. Roe, J.H. and Barnum, G.L., J. Nutrition. 11, 359, (1936).

48. Gould, B.S. and Shwachman, H., J. Biol. Chem. 151, 439, (1943).

49. Todhunter, E.N., McMillan, T.J. and Ehmke, D.A., J. Nutrition. 42, 297, (1950).

50. Langham, M., J. Physiol. (London) 111, 388, (1950).

51. Patterson, J.W. and Mastin, D.W., Amer. J. Physiol. 167, 119, (1951).

52. Matusis, I.I., Biokhimiya. 16, 420, (1951). Cited in Chem. Abst. 46, 2156i, (1952).

53. Kenyon, J. and Munro, N., J. Chem. Soc. 1948, 158.

54. Pecherer, B., J. Amer. Chem. Soc. 73, 3827, (1951).

55. deRitter, E., Cohen, N. and Rubin, S.H., Science. 113, 628, (1951).

56. Panteleeva, N.S., Trudy Leningrad Obshchestva Estestvoispytatelei, Otdel. Fiziol. i Biokhim. 69, No. 5. 143, 1950. Cited in Chem. Abst. 46, 4093h, (1952).

57./

57. Lloyd, B.B., J. Physiol. (London). 112, 49P., (1951).

58. Lloyd, B.B. and Parry, H.V., J. Physiol. (London). 126, 54P., (1954).

59. Bhattacharya, S.K., Ph.D. Thesis, Edinburgh University, 1954.

60. Patterson, J.W., Endocrinology. 45, 344, 1949.

61. Patterson, J.W., J. Biol. Chem. 183, 81, (1950).

62. Patterson, J.W., Science. 111, 724, (1950).

63. Patterson, J.W., Amer. J. Physiol. 165, 61, (1951).

64. Patterson, J.W. and Lazarow, A., J. Biol. Chem. 186, 141, (1950).

65. Patterson, J.W., Proc. Soc. Exptl. Biol. Med. 83, 850, (1953).

66. Patterson, J.W., Proc. Soc. Exptl. Biol. Med. 78, 758, (1951).

67. Merlini, D., Boll. soc. ital. biol. spr. 26, 1005, (1950).

68. Princiotto, J.V. J. Clin. Endocrinol. 11, 775, (1951).

69. Banerjee, S., Belavady, B. and Mukherjee, A.K., Proc. Soc. Exptl. Biol. Med. 83, 133, 1953.

70. Hughes, C.O., Swenson, M.J., Underbjerg, G.K.L. and Hughes, J.S., Science. 116, 252, (1952).

71. Owen, J.A., Iggo, B. and Horn, D.B., Nature. 174, 701, (1954).

72. Mindlin, R.L. and Butler, A.M., J. Biol. Chem. 122, 673, (1937-38).

73. Bessey, O.A., J. Biol. Chem. 126, 771, (1938).

74. Roe, J.H. and Kuether, C.A., J. Biol. Chem. 147, 399, (1943).

75. Bolin, D.W. and Book, L., Science. 106, 451, (1947).

76. Hagedorn, H.C. and Jensen, B.N., Biochem. Z. 137, 92, (1923).

77. Bessey, O.A. and King, C.G., J. Biol. Chem. 103, 687, (1933).

78. Daoud, K.M. and El Ayyadi, M.A.S., Biochem. J. 30, 1280, (1936).

79. Bowman, D.E. and Muntwyler, E., Proc. Soc. Exptl. Biol. Med. 35, 557, (1936-37).

80./

80. Sure, B., Theis, R.M. and Harrelson, R.T., J. Biol. Chem. 129, 245, (1939).

81. Sayers, G., Sayers, M.A., Liang, T.-Y., and Long, C.N.H., Endocrinology, 37, 96, (1945).

82. Dury, A., Amer. J. Physiol., 160, 75, (1950).

83. Ronzoni, E. and Reichlin, S., Amer. J. Physiol. 160, 490, (1950).

84. Oster, H.L., Kretchmar, A.L. and Bethel, F.H., Proc. Soc. Exptl. Biol. Med. 84, 470, (1953).

85. Todhunter, E.N. and McMillan, T.J., J. Nutrition. 31, 573, (1946).

86. Salhanick, H.A., Zarrow, I.G. and Zarrow, M.X., Endocrinology. 45, 314, (1949).

87. Sutton, T.S., Kaeser, H.E. and Hansard, S.L., J. Biol. Chem. 144, 183, (1942).

88. Reid, M.E., J. Nutrition. 35, 619, (1948).

89. Ludewig, S. and Chanutin, A. Endocrinology. 41, 135, (1947).

90. Sandberg, A.A., Eik-Nes, K., Samuels, L.T. and Tyler, F.H., J. Clin. Invest. 33, 1509, (1954).

91. Tyler, F.H., Schmidt, C.D., Eik-Nes, K., Brown, H. and Samuels, L.T., J. Clin. Invest. 33, 1517, (1954).

92. Reid, M.E., Proc. Soc. Exptl. Biol. Med. 68, 403, (1948).

93. Marcovich, A.W. and Marcovich, J.F. J. Lab. Clin. Med. 42, 681, (1953).

94. Dury, A. Proc. Soc. Exptl. Biol. Med. 82, 92, (1953).

95. Carey, M.M., Vollmer, E.P., Zwemer, R.L. and Spence, D.L. Amer. J. Physiol. 164, 770, (1951).

96. Levey, S. and Suter, B., Proc. Soc. Exptl. Biol. Med. 63, 341, (1946).

97. Sayers, G., Sayers, M.A., Liang, T. -Y., and Long, C.N.H., Endocrinology. 38, 1, (1946).